UNIVERSIDAD POLITÉCNICA DE MADRID

**ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,
ALIMENTARIA Y DE BIOSISTEMAS**

Functional analysis of two barley C1A
peptidases, HvPap-1 and HvPap-19, in
response to stress and in germination

TESIS DOCTORAL

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Licenciada en Biología

Máster en Agrobiotecnología

2020

Departamento de Biotecnología

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in response to stress and in germination.

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EL SECRETARIO

A mis padres

ACKNOWLEDGEMENTS

This Thesis has been performed in the Molecular Plant-Insect Interaction laboratory at the “Centro de Biotecnología y Genómica de Plantas (CBGP UPM-INIA)”, awarded with the accreditation as Centre of Excellence Severo Ochoa.

The Spanish “Ministerio de Economía y Competitividad (MINECO)” through a grant “Formación del Personal Investigador” (BES-2012-051962) associated to the project BIO2014-53508-R has supported this work.

A short-term stay in the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Gatersleben (Germany) was funded by MINECO (BES-2015-072192).

I greatly acknowledge Dr. Volodymyr Radchuk for bringing me the possibility to work in the “Assimilate Allocation and NMR” group at IPK in Gatersleben, learning new concepts and techniques and enjoying a wonderful experience.

I would like to especially acknowledge my thesis supervisors, Prof. Isabel Díaz and Pablo González-Melendi, and all the Plant-Insect Interaction group members for training, technical assistance and helpful discussion.

AGRADECIMIENTOS

Querido lector/a, si estás leyendo esto, es porque, finalmente, ¡esta tesis se ha llegado a escribir! Los que me conocéis bien sabéis que no ha sido un camino nada fácil y me habéis escuchado decir tropecientas mil veces eso tan dramático de “se acabó, lo dejo”. Parece ser que no ha sido así y esto es debido a la suerte que tengo de contar con algunas personas maravillosas que me habéis empujado a lo largo del camino.

Primero, agradecer a Isabel la oportunidad de poder embarcarme en este proyecto tan bonito. Por la ayuda prestada durante el proceso, los consejos aportados constantemente pensando en la mejor forma de abordarlo todo y la paciencia infinita. Gracias a Pablo por poner siempre el toque de cordura, el orden tan fundamental y por darle siempre esa vuelta de más que hace que las cosas queden “quasi perfectas”. He aprendido mucho contigo, tanto de microscopía como de ciencia. A Lola, me conoces desde mis tiernos inicios en la ciencia y, sin ti, no hubiera llegado hasta aquí. Espero seguir contando con tu guía en el futuro.

Gracias también a todos los miembros del labo 121, sobretodo y especialmente a Blanca, Estrella, Ana y Gara. Blanca, lo aprendí todo de ti, tienes una paciencia asombrosa, siempre dispuesta a ayudarme, a escuchar mis frecuentes dudas y a darme consejos que he valorado mucho, tanto profesional como personalmente. Estrella, pura energía y esa chispa divertida tan necesaria. Buenrollera, trabajadora y super mamá, eres un ejemplo. Anita Anita, quién nos ha visto y quién nos ve. Qué buenas tardes nos hemos pasado intentando hacer ciencia hasta horas interminables y arreglando el mundo con nuestras charlas infinitas. Bueno, y con alguna que otra foto divertida también. Eres toda una luchadora, te va a ir genial. ¡Garis! Mi hermana pequeña del CBGP. Qué buena eres y qué risas jaja. Muchas aventuras fuera y dentro del labo en poquito tiempo, y las que nos quedan. Merche, gracias por tu ayuda y tus consejos, seguro que te va fenomenal en tu nueva etapa. Manu, siempre dispuesto a aportar nuevas ideas, posibles explicaciones y soluciones a los desastres inminentes. Gracias también a todos los demás miembros del 121 que acabáis de llegar hace poquito. Dairon, Fany, Irene y Alejandro, hemos coincidido menos tiempo, os deseo lo mejor en ésta aventura. Alejandro, no dejes de proveer al mundo de mandarinas y tus ocurrencias, ambas son muy necesarias.

No puedo dejarme a todo el personal tan fantástico con el que cuenta el CBGP y que, sin ellos, las investigaciones no serían posibles. Chendo, eres de lo mejorcito que hay, tan trabajador y siempre dispuesto a ayudar y salvar mis cebadas de las catástrofes del invernadero. Nos quedan aún unos pinchos por la calle Van Dyck. María de esterilización, qué bien sabes cuidarnos.

Mención muy especial a René. Empezamos siendo colegas de humor malo malísimo, pero es que has sido mi postdoc por excelencia (de cuántos desastres me has ayudado a salir) y un gran amigo. Cuánto he aprendido (incluso de baile) y sigo aprendiendo contigo, y cuántas risas mientras tanto. Nos quedan muchos chistes oscuros y alguna que otra cerveza.

A Marta, Laura, Mar, Jose y Miguel del 127. Por toda la ayuda, los consejos, los cafés y el buen humor que tenéis.

A Julián, Loreto, Gerardo, Laura y Noemí. No hemos coincidido hasta mi última etapa, pero qué grato ha sido conoceros. Sois un grupo divertido a la par que interesante. Julián, Gerardo, tenemos muchas culturas y etnias que analizar en futuras charlas.

A todos los “viejas glorias” que nos habéis ido dejando por el camino. Alex, Marta, Castor, Bea, Rosario, Mario. Qué grandes momentos y qué grandes “Jornadas” con los aperitivos a cargo de becarios, unos buenos karaokes improvisados y nuestra querida “AJÍ”.

A todos los miembros del 285 con los que he tenido la suerte de coincidir: Paul, Saray, Sandra, Clara, Andrea, Sofía, Alba (Chufi), Emilia y Chechu. Junto con las nuevas incorporaciones en Bioinformática: Jaime, Álvaro (Kaixo), Lisa, Pablo, Ana (Peke) y a Elena, que la adoptamos. Me encanta el grupo que hemos formado y nuestras escandalosas reuniones tanto dentro como fuera del CBGP. Gracias por ser además grandes amigos, por el apoyo infinito y por las risas siempre necesarias. Por cierto, ya va tocando otro vermou-th-torero.

Por supuesto, por supuestísimo, a mi querida “LaPeñita”. Qué hubiera sido de mí sin vosotros. Victor (VickDaddy, Victorsito, VickyYi), aquella cena de navidad nos unió y en modo pack hasta ahora (junto con tu pelazo). Ya sabes lo que significas para mí. Seguiré siendo tu proveedora oficial de farinatos hasta que tu colesterol diga basta. JPaul (JenmPol, JeanPollito, Peruvian... jaja) mi “amigo peruano” favorito, te quiero igual, aunque no te guste el farinato. Nos quedan unos Viñas por ahí eh. Lau, “Fresi”, por todo en general (ya sabes tú). Sigue viviendo la vida como la vives, me encanta. Patri, uf, qué intensidad en tan poco tiempo. Gracias por esa gran amistad que seguimos manteniendo en la que no hay distancias, por las cervezas por Skype tan necesarias, las ferias de libro, los conciertos y las charlas infinitas. Habéis sido mi soporte vital dentro en el CBGP y fuera, ha habido grandes viajes, conciertos y festivales. Nos queda mucha música por bailar.

A los “míos” de toda la vida, Alex, Bea, Sara, Noe, Silvia y Patri. Mis “Montijos”. Cuánto me encanta teneros en mi vida y contar con vosotros desde siempre. Sin vuestro apoyo no hubiera llegado hasta aquí.

A Leyre, ¡cómo no! Por todo, porque ya nos entendemos tú y yo, y porque en verdad no hace falta que te diga nada. A Teresa, porque eres de las amigas más valientes que tengo y por cómo me ves siempre. A David, Mex, alias “Totopi”. Por acogerme en Madrid cuando aún no tenía casa, por todo el apoyo y nuestras magníficas quedadas. Nos vemos en nuestro mexicano de confianza.

A Bea, Sergio y Shira. Sois la mejor familia “postiza” que me podía tocar y habéis sido fundamentales en estos años. Aún nos quedan muchos restaurantes con comida rica que probar y rutas que hacer para bajarla.

A Tote y Mario, sois maravillosos. A Edu, gracias por seguir haciéndome rabiar de vez en cuando, sabes cómo tirar de mí después de tantos años. A María y Néstor, por ser mi primera familia en Madrid.

A Aleksandra (Ola), por ser mi familia polaca en Alemania. ¡Qué hubiera sido de mí sin ti! Y todo lo que me enseñaste. Eres una gran profesional, tienes una casa en España cuando quieras. A Matthias (The Kiddo) y todo el grupo AAN, me enseñasteis mucho, sobretodo otra forma de ver las cosas. A Volodya y Ludjmila, por darle un sentido a mi historia. A Matías y Eva y todo ese grupazo que hicimos en el club del IPK.

A mis “Biolo-cos”. Bea y Mery, sois fantásticas, siempre me aportáis amor y serenidad. Mariolis, gran político, mejor persona, leal amigo. Jorge y Ana, qué bueno teneros. Jose y Kasteel, los Zipi-Zape. Kasteel, ya tocan unas buenas “Kastichas”. Guille, mi mano izquierda, lo sabes todo ya. Que sigan las bodas, los bebés y lo que sea, pero juntos.

A mis padres. Por vuestra bondad, comprensión e incalculable apoyo en todos los momentos difíciles. Porque soy como soy por vosotros, porque sois las personas más valientes del mundo y me habéis enseñado a ser fuerte. Porque siempre estáis ahí y porque las gracias se quedan cortas. Samuel, siempre serás mi enano pequeñito. Al pequeño Jagger, qué bonito tenerte en nuestras vidas.

A Álvaro. Nos conocimos desde bien pequeños, nos volvimos a encontrar de adolescentes y perdimos el contacto en la Universidad, pero no puedo estar más agradecida de que el hilo rojo nos uniera de nuevo, para hacer un nudo bien fuerte. Por todo lo que nos queda por recorrer y porque sigamos cumpliendo sueños juntos. No podía estar más feliz de compartir mi vida contigo.

ABBREVIATIONS

A: Aleurone

AB: Antibody

ABA: Absciscic Acid

AGG3: Arabidopsis G Gamma Subunit 3 Protein

AMC: Amido Methyl Coumarin

amiRNA: Artificial microRNA

APS: Ammonium PerSulphate

BiFC: Bimolecular Fluorescent Complementation

BSA: Bovine Serum Albumine

CaM: Calmodulin

CCD: Charged-Coupled Device

CDKs: Calcium Dependent Protein Kinases

cDNA: Complementary Deoxyribonucleic Acid

CE-ESI-TOF MS: Capillary Electrophoresis ElectroSpray Ionization-Time-Of-Flight Masses Spectrophotometry

CM: Complete Medium

CysProt: Cysteine Proteases

d: Days

daf: Days After Flowering

DAMPs: Damage-Associated Molecular Patterns

Dep1: Dense and Erect Panicle 1

DG: Developing Grains

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic Acid

Em: Embryo

ET: Ethylene

ETI: Effector-Triggered Immunity

FT – NIR: Fourier Transform Near Infrared

g: Gravitational force

GA: Gibberellic Acid

Gb: Gigabase

GC: Grain Coat

GFP: Green Fluorescent Protein

gs: Stomatal Conductance

h: Hours

hai: Hours After Imbibition

HAMPs: Herbivore-Associated Molecular Patterns

HPT: Hygromycin Phosphotransferase

HR: Hypersensitive Response

HSD: Honestly Significant Difference

HvPDIL1-1: Protein Disulfide Isomerase

JA: Jasmonic Acid

Ja-Ile: Jasmonic Isoleucine

KD: Knock Down

***m/z*:** mass-to-charge ratio

M: Molar

MAMPs: Microbe Associated Molecular Patterns

MAPK: Mitogen-Activated Protein Kinases

Mbar : Mili bares

MHz: Mega Hertz

min: Minute

mRNA: messenger RNA

MS: Mass Spectrophotometry

ms: Miliseconds

MW: Molecular weight

NMR: Nuclear Resonance Magnetic

NO: Nitric Oxide

OPDA: 12-oxo-phytodienoic acid

P: Pericarp

PAMPs: Pathogen-Associated Molecular Patterns

PB: Protein Bodies

PBS: Phosphate Buffered Saline

PCD: Programmed Cell Death

PhyCys: Phytocystatins

PRR: Pattern Recognition Receptors

PSV: Protein Storage Vacuoles

PTI: PAMP-Triggered Immunity

QTL: Quantitative Trait Loci

RBOHs: Respiratory Burst Oxidase Homologs

RER: Rough Endoplasmic Reticulum

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rpm: Revolutions per Minute

RT-qPCR: Reverse Transcription quantitative Polymerase Chain Reaction

s: Seconds

SA: Salicylic Acid

SAGE: Salicylic Acid Glucosyl Ester

SAM: Shoot Apical Meristem

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SE: Standard Error

SEM: Scanning Electron Microscopy

SerProt: Serine Proteases

SNK: Student Newman Keuls

SPCP2: Sweet Potato CysProt

T: Testa

TAE: Tris Acetate EDTA

TD - NMR: Time Domain Nuclear Resonance Magnetic

UV: Ultra Violet

v / v: Volume / Volume

V: Voltage

VB: Vascular Bundle

w / v: Weigh / Volume

WT: Wild Type

ZFR-AMC: N- carboxybenzoxy - Phe-Arg-7-amido-4-methyl coumarin

Z-R-AMC: N- carboxybenzoxy - Arg- amido-4-methyl coumarin

ZRR-AMC: N- carboxybenzoxy - Arg-Arg-7-amido-4-methyl coumarin

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RESUMEN

La degradación y movilización de proteínas desde tejidos maduros o sometidos a estrés hasta los órganos en desarrollo o sumidero son procesos metabólicos inherentes a la fisiología de las plantas. La proteólisis es responsable de la homeostasis celular y el reciclaje de componentes celulares y de nutrientes, y se basa en la degradación de proteínas a través de enzimas hidrolíticas, entre las que la familia C1A de Cisteín-Proteasas (CysProt) tiene un papel muy relevante. En cebada, especie utilizada de esta tesis, se han identificado 42 genes que codifican peptidasas C1A, pero solo se ha estudiado la función de algunos miembros de esta familia. Dilucidar qué proteasas participan en un determinado proceso fisiológico, cuáles son sus dianas, y cuál es su impacto en la planta ha sido el objetivo de este trabajo. Se ha utilizado el sistema cebada-proteasas C1A para identificar las proteasas implicadas en la respuesta a estrés mediada por sequía y en la germinación de las semillas, con el fin de estudiar su impacto sobre el crecimiento de la planta y la calidad del grano.

La cebada (*Hordeum vulgare* L.) es un cultivo de interés que ocupa el cuarto lugar entre los cereales después del trigo, maíz y arroz, según la superficie cultivada a nivel mundial. Se utiliza como alimento para ganado y consumo humano, para la fabricación de bebidas alcohólicas y la producción de biocombustibles. Además, es una especie considerada modelo en el escenario de cambio climático por su capacidad de adaptación ecológica.

Tras analizar los patrones de expresión de todos los genes de la familia C1A en hojas de cebada sometidas a sequía, y estudiar los perfiles transcriptómicos de la misma familia multigénica durante la germinación de la semilla, se seleccionaron dos genes, *HvPap-1* y *HvPap-19*, que codifican catepsinas tipo F y B, respectivamente, por sus altos niveles de expresión en ambos procesos fisiológicos, para realizar un estudio profundo de su función. Además de la caracterización molecular de ambos genes y la validación de su expresión, se generaron líneas de expresión reducida (*knock down*) para analizar los cambios morfológicos y funcionales que podrían producirse durante el estrés hídrico y en la germinación. De los resultados obtenidos, se puede concluir que la ausencia, incluso parcial, de las proteasas *HvPap-1* y *HvPap-19* alteraba la estructura foliar modificando el grosor de las cutículas y la apertura de los estomas, lo que les confería nuevas propiedades de defensa frente a la sequía y además, al ataque de patógenos y fitófagos. Asimismo, se detectaron variaciones en la composición de las semillas que modificaban los patrones temporales de germinación. Estos resultados abren nuevas líneas de investigación y potenciales aplicaciones que podrían contribuir a mejorar la cosecha de este cereal y la calidad y rendimiento del grano.

ABSTRACT

Protein breakdown and mobilization from old or stressed tissues to growing and sink organs are some of the metabolic features associated with the plant physiology. Thus, the proteolysis is a physiological process essential for cell homeostasis and cell components and nutrient recycle, based on the degradation of proteins mediated by hydrolytic enzymes. The C1A cysteine-protease (CysProt) family has a relevant role in these processes.

In barley, the plant species studied in this thesis, 42 genes encoding C1A peptidases have been identified but up to now, it is limited the number of members functionally described. Deciphering what proteases are involved in a specific physiological process and what is their impact in the plant have been the main objectives of this work. This thesis has focused on the barley-C1A CysProt system as a promising model to identify the protease members involved in the response to drought as well as in the grain germination, to then analyze their impact on the plant growth and grain quality.

Barley (*Hordeum vulgare* L.) is cereal crop of interest that occupies the fourth position after wheat, maize and rice based on the number of sown worldwide hectares. It has important uses for animal feed and human food, brewing industry, and biofuel production. Besides, it is considered a model plant in the climate change scenario due to its ability of ecological adaptation.

We tested the expression levels of the whole CysProt family members in barley leaves after water deprivation, and analyzed the transcriptomic profile of this protease family in a microarray performed in grain development and germination. Among the differential expressed genes common in the two physiological processes, *HvPap-1* and *HvPap-19* encoding a cathepsin F- and B-like, respectively, showed the highest expression levels, and therefore were selected for a deeper functional analysis. Besides the molecular characterization of these genes and the validation of their expression, knock down transgenic lines were generated to analyze potential morphological or functional changes associated with water deprivation of grain germination. Results indicated that the absence, even partial, of *HvPap-1* and *HvPap-19* proteases modified the foliar structure altering leaf cuticle thickness and stomata aperture and conferred new defense properties against drought and pathogen and phytophagous attack. In addition, changes in the grain composition that altered the timing of germination were also observed. These results open new research lines and may contribute to improve the barley yield and the grain quality.

GENERAL INTRODUCTION

1. BARLEY AS A MODEL PLANT IN BIOTECHNOLOGY.

Barley is a grass cereal of the Poaceae family, also including other species of agricultural and economical interest such as wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.). Its domestication is traced back to the onset of the agriculture in the Fertile Crescent during the development of the Neolithic culture (Takahashi 1955). The wild ancestor of cultivated barley was *Hordeum spontaneum* K. Koch also known by its synonym *Hordeum vulgare* subsp. *spontaneum* (K. Koch) (https://wcsp.science.kew.org/namedetail.do?name_id=419529), a two-rowed cultivar (cv) (Weiss and Zohary 2011; Mascher *et al.* 2016). The six-rowed trait of *H. vulgare*, which yields more seeds per spike, was developed throughout that process of domestication (Komatsuda *et al.* 2007; Pourkheirandish and Komatsuda 2007; Sakuma *et al.* 2011). Cultivated barley is a diploid species with a large haploid and sequenced genome of 5.1 gigabases (Gb), distributed in 7 chromosomes ($2n = 14$) (Mayer *et al.* 2012).

According to the last FAO (Food and Agriculture Organization of the United Nations) statistic data (FAOSTAT 2019) barley grains are, among cereals, the most consumed after wheat, corn and rice. Their main end-uses are animal feed and forage, and malt production for the elaboration of alcoholic beverages like whisky and beer. In world areas where is not possible to grow other cereals, about 5% of the annual harvest of barley is destined to human consumption (Tricase *et al.* 2018; Giraldo *et al.* 2019). Barley grains provide nutritional benefits due to their high content in soluble fiber (helping digestion) and β -glucans (to maintain an adequate cholesterol balance in the blood) (Gani *et al.* 2012; Shah *et al.* 2017). They also have an important use in molecular farming as biofactories for the production of human therapeutic proteins or animal vaccines (Horvath *et al.* 2000; Mrízová *et al.* 2014). In addition, their polysaccharide content make barley grains a source to elaborate biofuels and fuel pellets (Hicks *et al.* 2014).

Barley is widely distributed due to the variety of cultivars (winter and spring types) adapted to different climatic areas. This crop can grow under drought, high salinity, high temperature or low fertility soil conditions (Gürel *et al.* 2016). In Spain, the production of barley is the

highest among cereals, followed by wheat, maize and rice (data from 2017, Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente, www.mapa.gob.es).

The global climate change, driven by the greenhouse effect caused by the accumulation in the atmosphere of gases such as CO₂, will bring about less rain fall and higher temperatures, which together with the rise of the World population, and higher salinity and lower fertility soils can threaten feed and food security in the forthcoming years (Arenas-Corraliza *et al.* 2019). This challenge has to be tackled multidimensional, including the concern and actions from the national governments, stakeholders and the whole societies, and biotechnological approaches for the adaptation of the cultivars without affecting crop quality. The trend of barley production in Southern Europe is becoming stagnant, which can be related to this scenario (Dawson *et al.* 2015). Then, the identification of traits of resistance to biotic and abiotic stresses is required to counteract the foreseeable consequences of climate warming.

Barley is a model cereal due to its diploid genome, low chromosome number and ease of cross-breeding (Dawson *et al.* 2015). To find out strategies for barley improvement a number of molecular and genomic tools are nowadays available such as DNA and RNA sequencing, identification of epigenetic modifications, localization of quantitative trait loci (QTL) and transformation of genes of interest (Honsdorf *et al.* 2014; Dawson *et al.* 2015). The earliest report of barley transformation by particle bombardment is dated to 1994 (Wan and Lemaux). Later, barley transgenic plants were generated by co-culture of immature embryos with *Agrobacterium* (Tingay *et al.* 1997). A major breakthrough was the combination of *Agrobacterium*-mediated transformation with the technology of double-haploids, using microspores in the case of the cultivar Igry (Kumlehn *et al.* 2006) or immature embryos for Golden Promise (Coronado *et al.* 2005, Hensel *et al.* 2009), and subsequent embryogenesis to yield spontaneously homozygous plants in a shorter time than other methods and without colchicine treatment.

Golden Promise is a Scottish spring two-rowed barley malting cultivar, which is transformation amenable (Wendt *et al.* 2016). It was generated by gamma-ray induced mutagenesis of the cultivar Maythorpe to introduce the trait of short and stiff culm, suitable for agriculture in Scotland, into a malting background. The *ari-e.GP* mutation confers the semi-dwarf phenotype of Golden Promise. This mutation was identified with a loss-of-function of the gene *HvDep1*, ortholog to rice *Dep1*, which encodes the gamma subunit of

the AGG3 type of heterotrimeric G proteins (Wendt *et al.* 2016). At present, this cultivar is no longer used for malting purposes and has become a model for barley transformation. Golden Promise was used to generate the transgenic plants for the experiments presented in this Doctoral Thesis.

2. STRUCTURE AND CONTENT OF THE BARLEY GRAIN

The barley mature grain or caryopsis is a dry and non-dehiscent fruit protected by enclosing coats (Rodríguez *et al.* 2015) (Fig. 1). The outermost is the hull formed by two distinct covers, the ventral lemma and the dorsal palea. The next layer inwards is the pericarp (fruit coat), which is fused with the testa (seed coat). The aleurone, which confines the starchy endosperm, is a tissue with 2-3 layers of live cells containing protein storage vacuoles (PSV) and lipid bodies (Arcalis *et al.* 2014). Upon germination it secretes hydrolases to break down the starch, lipids and proteins stored in the seed. The starchy endosperm is the largest part of the grain and it is formed by triploid cells that undergo programmed cell death (PCD) in the latest stages of grain development (Sreenivasulu *et al.* 2006; Domínguez and Cejudo 2014). In its mature stage, it accumulates starch granules within a protein matrix. The subaleurone, beneath the aleurone, is rich in proteins and contains less starch than the inner starchy endosperm (Jääskeläinen *et al.* 2013). The embryo axis comprises the radicle, protected by the coleorhiza, and the shoot apical meristem (SAM) and primary leaves (plumule) surrounded by the coleoptile. The scutellum (cotyledon) transfers nutrients from the endosperm to the embryo (Lopato *et al.* 2014).

The exit from dormancy occurs when the dry seed uptakes water (imbibition), triggering germination. Imbibition is followed by the growth and elongation of the embryo axis and the protrusion of the radicle out of the grain coat. During germination the gibberellic acid (GA) synthesized in the embryo is released and delivered through the scutellum to the aleurone where the synthesis of hydrolases is induced (Fig. 1). These enzymes are secreted into the endosperm to mobilize the nutrients stored there, which are then absorbed by the scutellum and transported to the embryo in order to sustain the growth and development of the plantlets until photosynthesis is established (Ma *et al.* 2017; Díaz-Mendoza *et al.* 2019). Starch, the major component of the grain, is broken down by α - and β -amylases.

Triacylglycerol molecules stored in lipid bodies are hydrolyzed by lipases into glycerol and fatty acids, which in turn are degraded by β -oxidation. Then, the glyoxylate cycle transforms acetyl-CoA in succinate, producing malate in the mitochondria, which is used for the cytosolic reactions of gluconeogenesis. Proteins represent the 8-12% of the grain dry weight and provide a source of nitrogen and sulfur. They are hydrolyzed by cysteine and serine proteases (CysProt and SerProt, respectively) into short peptides and amino acids during germination (Ma *et al.* 2017; Roustan *et al.* 2018; Díaz-Mendoza *et al.* 2019).

Barley grains contain three protein fractions with different solubility and extraction methods (Osborne, 1895): albumins (water), globulins (salt solutions) and prolamins (aqueous alcohol). Globulins represent 10-20% of the total grain proteins. Storage globulins (7S vicilin-type and 11S legumain-type) are mainly present in the aleurone and the embryo (Gubatz and Shewry 2011). Others, such as β -amylase, are enzymes. Prolamins, named hordeins in barley, account for 50–80% of the protein content of the grain. According to their MW, hordeins are classified in four types: D (<100 KDa), C (55-75 KDa), B (35-46 KDa) and Y (>20 KDa). The major fractions are sulfur-rich B-hordein (70–80%) and sulfur-poor C-hordein (10–20%) (Tapia-Hernández *et al.* 2019). Hordeins, with different percentages among them, are rich in glutamine and proline, which confer their characteristic solubility, and poor in lysine. Cysteines form disulfide bonds within or among hordein polipeptides, promoting protein folding and aggregation. An N-terminal signal peptide directs the co-translational translocation of hordeins into the lumen of the rough endoplasmic reticulum (ER), where they form aggregates into protein bodies (PB) in the subaleurone and starchy endosperm. Hordeins (D- and B-) are more abundant in the latter and are located, together with protein disulfide isomerase (HvPDIL1-1), in a protein matrix at the periphery of starch granules in developing grains (Roustan *et al.* 2018). PB develop in PSV either via Golgi dependent or independent routes (Arcalis *et al.* 2014). It has been suggested that the latter could occur by an autophagy-like deliver of the PB to the vacuole (Herman and Larkins 1999). Inactive proteases follow the same secretory pathway where they colocalize with their targets. When germination is triggered these enzymes become active and together with *de novo* synthesized proteases hydrolyze the storage proteins.

Fig. 1. Longitudinal section of a barley grain. During germination, gibberellic acid (GA) induces gene expression of hydrolases in the aleurone to break down starch, lipids and proteins.

3. PLANT PROTEASES

Proteases are enzymes broadly distributed in prokaryotes and eukaryotes that hydrolyze peptide bonds within polypeptides. Proteolysis is essential for post-translational processing, elimination of damaged or aggregated proteins, protein turnover and recycling of amino acids, protein removal of regulatory proteins or food digestion, among other pivotal functions for the maintenance of cell homeostasis (Beers *et al.* 2000; Grudkowska and Zagdanska, 2004; Habib and Fazili, 2007). Higher plant genomes encode hundreds of proteases that participate in many processes such as mobilization of storage proteins during grain germination, development, cell death, mitosis and meiosis, embryogenesis, seed coat formation, stomata development, response to environmental stresses, senescence and defense (van der Hoorn *et al.* 2008). Most proteolytic activities rely on vacuolar proteases (Müntz 2007), but proteolysis can also occur in the nucleus, cytosol or organelles such as mitochondria, peroxisomes and plastids (Beers *et al.* 2000; van Wijk 2015).

Proteases that cut off internal bonds within polypeptides are called endoproteases. Exoproteases, either aminopeptidases or carboxypeptidases, cleave peptide bonds at the N-terminal or C-terminal position, respectively (Kîdrîc *et al.* 2014). MEROPS (<https://www.ebi.ac.uk/merops/>) is an updated proteases database that establishes a hierarchical classification where the enzymes are clustered into families, which in turn are

grouped in clans (Rawlings *et al.* 2018). Proteases within a family share similar sequences and a clan contains resembling tertiary structures. The first letter is the identifier of clans and families and denotes the catalytic type, using the following code: A stands for aspartic proteases, C for cysteine proteases, G for glutamic proteases, M for metalloproteases, P for proteases of mixed catalytic type, S for serine proteases, T for threonine proteases, N for asparagine lyase proteases, and U for peptidases of unknown catalytic type. The letter I corresponds to proteinaceous inhibitors.

3.1. C1A cysteine proteases and their inhibitors.

According to the structure-based classification of peptidases of the MEROPS database, the subfamily of C1A CysProt, also known as papain-like CysProt, is one of the most abundant in plants (Martínez *et al.* 2012; Rawlings *et al.* 2018). Martínez and Díaz (2008) grouped C1A CysProt as cathepsin L-, B-, H- and F- types, based on their homology to mammalian lysosomal proteases. The 42 C1A CysProt identified in barley are distributed as follows: 35 L-, 3 B-, 1 H- and 3 F-like. These proteases form globular proteins stabilized disulfide bonds with a catalytic triad (Cys, His, and Asn) and a Gln residue, which is essential for their active conformation (Martínez and Díaz, 2008; Cambra *et al.* 2012a).

Fig. 2. General structure of C1A CysProt. SP, signal peptide (grey). Pro-peptide region (black): consensus motif GxNxFxD; non-contiguous ERFNIN/ERFNIQ (depending on the cathepsin type). Mature protease (orange): residues Cys, His and Asn of the active site; GCNNG like-motif common to all CysProts; KDEL, an ER return signal present in some soluble L-like C1A proteases; Pro-rich and granulin domains (Cambra *et al.* 2012a, b). Adapted from Velasco-Arroyo, 2017.

C1A CysProt are synthesized as inactive proenzymes with N-terminal propeptides that have to be removed to become catalytically active. Propeptide cleavage requires an acidic pH and the autoproteolysis or hydrolysis by other proteases. Propeptides can also be located within the polypeptide chain and/or at the C-terminal position of the zymogen (Brix *et al.* 2014). Propeptide hydrolysis secures that enzyme activation occurs in the correct place and/or time to avoid undesired side-effects. Free propeptides of cathepsins B- and L-like are potent inhibitors of peptidases by binding to the active site of the protease in a reverse orientation as the substrate to block its access (Guay *et al.* 2000; Schilling *et al.* 2009). C1A propeptides contain the consensus motif GNFD required for the correct processing of the protease and a noncontiguous ERFNIN signature in cathepsin L- and H-like or an ERFNAQ variant in cathepsin F-like. This signature is not present in cathepsin B- like proteases. Some L-like C1A CysProt have a C-terminal KDEL/HDEL that identifies soluble ER resident proteins (Fig. 2) (Beers *et al.* 2000; Grudkowska and Zagdanska 2004; Martínez and Díaz 2008; Cambra *et al.* 2012a). In addition, some cathepsin L-like also contain a C-terminal extension which includes a Pro-rich region and a granulin domain with a high homology to animal proteases (Yamada *et al.* 2001). This domain may be involved in the protease solubility (Beers *et al.* 2000).

C1A CysProt participate in different processes such as senescence, abscission, autophagy, programmed cell death, protein mobilization in seeds and tubers (van der Hoorn 2008; Martínez *et al.* 2012; Díaz-Mendoza *et al.* 2016 a,b; Sueldo and van der Hoorn 2017; Bárány *et al.* 2018). These proteases are also up-regulated in response to abiotic (darkness, drought or salt) or biotic stresses (pathogens and pests) (Rabbani *et al.* 2003; Shindo and van der Hoorn 2008; Parrot *et al.* 2010; Guo and Gan 2012; Roberts *et al.* 2012; Díaz-Mendoza *et al.* 2014; Kempema *et al.* 2015; Velasco-Arroyo *et al.* 2016; Díaz-Mendoza *et al.* 2017; Thomas and van der Hoorn 2018; Wang *et al.* 2018; Gómez-Sánchez *et al.* 2019).

C1A CysProt are inhibited by low molecular weight (MW) proteins called phytocystatins (PhyCys) (Martínez and Díaz 2008). Those of higher MW, approximately 23 KDa, can also inhibit C13 CysProt (so-called legumain-like). The 13 PhyCys identified in barley regulate the activity of CysProt in several processes, such as drought stress, protein turn-over and defense mechanisms (Martínez *et al.* 2009, 2012; Benchabane *et al.* 2010; Carrillo *et al.* 2011; Santamaría *et al.* 2012a; 2018b; Velasco-Arroyo *et al.* 2018a,b). CysProt and PhyCys colocalize within the endomembrane system as demonstrated by bimolecular fluorescent

complementation (BiFC) (Martínez *et al.* 2009). The interaction between PhyCys and CysProt is reversible.

3.2. C1A cysteine proteases involved in germination events

Members of the C1A and C13 CysProt and SerProt (Mikkonen *et al.* 1996; Hara-Nishimura *et al.* 1998; Prabucka and Bielawski 2004; Sreenivasulu *et al.* 2008; Shi and Xu 2009; Radchuk *et al.* 2011, Cambra *et al.* 2012b; Díaz-Mendoza *et al.* 2016, 2019) mainly accomplish the mobilization of storage proteins during barley germination. Two-dimensional electrophoresis revealed 42 proteases involved in barley grain germination of which 27 were CysProt, 8 SerProt, 4 aspartic proteases and 3 metalloproteases (Zhang and Jones 1995). Later, a transcriptomic analysis identified 20 up-regulated C1A CysProt (Close *et al.* 2004). Four L-like CysProt genes were expressed in embryos under germination, of which the higher levels of mRNA corresponded to *HvPap-10*, followed by *HvPap-6*, *HvPap-4*, and *HvPap-17* (Martínez *et al.* 2009). Also, cathepsins B- (*HvPap-19* and *HvPap-20*), H- (*HvPap-12*) and F-like (*HvPap-1*) were detected in germinated aleurone and embryo (Sreenivasulu *et al.* 2008; Cambra *et al.* 2012b)

4. ABIOTIC AND BIOTIC STRESS

Plants are exposed to unfavorable environmental conditions, anthropogenic actions against the environment and the challenge of pathogens and herbivores that interfere with their growth and threaten their survival, which in the case of crops lead to significant losses in agricultural productions. Abiotic stress can be triggered by physical and chemical agents such as water (excess or shortage), cold, heat, salinity, light or metals when a reference state is exceeded, compromising physiological processes (Kranner *et al.* 2010; Kîdrick *et al.* 2014). Also, the biotic stress, an interaction of plants with pathogens and pests, has a negative impact on agriculture since the host has to divert resources to defend itself and survive (Foyer *et al.* 2016; Misas-Villamil *et al.* 2016). Synergies of different stresses can boost these deleterious effects. For example, higher temperatures in the current context of climate change bring about a better performance of pathogens and pests and also are responsible

of drought periods. Upon perception of stress, the plants respond by activating signal transduction mechanisms that end up in gene expression of mainly structural proteins and metabolic enzymes to adapt and protect themselves from the hostile conditions (Zandalinas *et al.* 2017).

4.1. Drought stress and cysteine proteases

Drought is considered to be one of the most critical threats to agriculture worldwide and is responsible for the largest losses of crops. Plants defend themselves against adverse conditions, and in particular to drought, by activating an orchestrated and highly regulated molecular network that triggers biochemical, physiological, and morphological changes to minimize damage. Under drought stress, stomata are closed to prevent transpiration, which limits CO₂ uptake, reduces photosynthesis, and subsequently impairs plant growth. Osmotically active compounds and protective proteins accumulate, and sink/source allocation is adjusted. Also, the shoot-to-root ratio decreases, and a deeper root system develops. The cuticle is a hydrophobic, two-layered structure formed by cutin, a network of esterified fatty acids embedded into the pecto-cellulosic cell wall, and outer cuticular waxes (esterified fatty acids and alcohols) deposited on top of the cutin. Under water limitation conditions the cuticle becomes thicker to protect the aerial part of the plant from water loss (Li *et al.* 2013; Joubès and Domergue 2018). It also reflects UV radiation to avoid DNA damage.

These events are regulated by changes in the hormone content, mainly by altering the abscisic acid (ABA) levels, which act as key regulator of the global process (Osakabe *et al.* 2014; Shi *et al.* 2016; Bi *et al.* 2017; Xue *et al.* 2017). Drought stress brings about premature plant senescence, which usually leads to a reduction in the canopy size and to a drop in the yield in annual crops. The impact is even more drastic when water deficit occurs during the reproductive or grain filling stages (Barnabas *et al.* 2008; Mahalingam 2017).

High protease activities are closely associated with plant responses to different stresses, particularly water deficit, and they enhance protein turnover required for metabolic processes and nutrient recycling (Khanna-Chopra *et al.* 1999; Beyene *et al.* 2006; Kîdrîc *et al.* 2014; Botha *et al.* 2017). Drought sensitive plants have a higher proteolytic activity than resistant ones (Beyene *et al.* 2006; Simova-Stoilova *et al.* 2010). The upregulation of C1A

CysProt genes is essential for protein breakdown in stress responses by triggering reorganization of metabolism, remodelling of cell protein compounds, degradation of damaged or unnecessary proteins and remobilization of nutrients (Rabbani *et al.* 2003; Parrott *et al.* 2010; Guo and Gan 2012; Roberts *et al.* 2012; Díaz-Mendoza *et al.* 2014; Wang *et al.* 2018). Zang *et al.* (2010) demonstrated that the overexpression of the *T. aestivum* CysProt (*TaCP*) gene conferred stronger tolerance to water deficit than the WT plants. Likewise, the ectopic expression of the sweet potato CysProt (*SPCP2*) gene in *Arabidopsis thaliana* L. altered plant growth and enhanced the tolerance to drought and/or salt stress (Chen *et al.* 2010). Conversely, CysProt may play the opposite role as it was shown by the increased sensitivity to drought stress and the altered phenotypic traits observed in transgenic *A. thaliana* plants constitutively expressing the sweet potato *SPCP3* CysProt gene (Chen *et al.* 2013). Barley knock down (KD) plants with reduced levels of *HvPap-1* and *HvPap-19* CysProt, KD Pap1 and KD Pap19 respectively, and the WT developed thicker cuticles under 14 d of drought than the watered controls (Gómez-Sánchez *et al.* 2019). KD Pap1 cuticles (both control and subjected to drought) were significantly thicker than the KD Pap19 and WT ones (Gómez-Sánchez *et al.* 2019). After drought the stomata pore area decreased in the WT vs. the control conditions, increased significantly in the KD Pap19 plants, and the results were intermediate for the KD Pap1 plants (Gómez-Sánchez *et al.* 2019).

PhyCys participate actively during abiotic stress response. Accumulating evidences suggested that cystatin over-expressing plants with less CysProt activity are more tolerant to water deprivation (Zhang *et al.* 2008; Quain *et al.* 2014; Tan *et al.* 2017). Recently, Velasco-Arroyo *et al.* (2018) have provided the first evidence about the effects of cystatins on plant behavior under a deficit-watering regime, by independently silencing two barley drought-induced cystatin genes, *Icy-2* and *Icy-4*.

4.2. Biotic stress and cysteine proteases

Plants have developed through evolution an innate immunity system. Pattern recognition receptors (PRR) localized in the plasma membrane bind elicitors from microorganisms (P/MAMPs, pathogen/microbe-associated molecular patterns) and herbivores (HAMPs, herbivore-associated molecular patterns) and also self-components released by the lysis of the host cells (DAMPs, damage-associated molecular patterns) (Dodds and Rathjen 2010;

Dangl *et al.* 2013; Bigeard *et al.* 2015; Santamaría *et al.* 2018b). The plant cuticle is an inert, smooth physical barrier that prevents the attachment of pathogens or pests on the surface of the aerial parts of the plants (Jenks *et al.* 1994; Schweizer *et al.* 1994, 1996). When the plant cuticle is damaged releases signals perceived by plants as elicitors that trigger the defence response (Schweizer *et al.* 1994; 1996). This recognition activates plant downstream defence mechanisms or PTI (PAMP-triggered immunity), which involve Ca^{2+} influxes into the cytosol and the depolarization of the plasma membrane. Ca^{2+} , as a secondary messenger, activates CDPKs (Calcium Dependent Protein Kinases), which in turn phosphorylate and activate RBOHs (Respiratory Burst Oxidase Homologs) to produce reactive oxygen species (ROS), and also calmodulin (CaM) that induces the synthesis of nitrogen oxide (NO). Then, the activation of intracellular MAPK (Mitogen-Activated Protein Kinases) signalling cascades leads to plant defence responses such as the deposition of callose, the synthesis of antimicrobial compounds, the release of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), the closure of the stomata to avoid the entry of pathogens and the activation of transcription factors to induce gene expression. During coevolution some pathogens and pests have developed effector molecules that suppress plant PTI. Plants can respond producing intracellular receptors (PRR proteins) that interfere with these effectors. This is known as effector-triggered immunity (ETI), which involves long term actions such as affecting development and fecundity of the phytophagous arthropods or the hypersensitive response (HR), (Santamaría *et al.* 2018a)

Proteases can be direct or indirect targets of microbial virulence elcitors/effectors (van der Hoorn 2008; Niño *et al.* 2014). Some examples of the C1A CysProt participation in plant defense against pathogens and pests are *Rcr3* and *PIP1* in *Solanum lycopersicum* L., *Lycopersicum esculentum* P. Mill. and *Solanum pimpinellifolium* (Phil.) Reiche, (Krüger *et al.* 2002; Tian *et al.* 2007; Lozano-Torres *et al.* 2012), *RD19* and *RD21* in *A. thaliana* (Bernoux *et al.* 2008; Shindo *et al.* 2012), *CathB* in *Nicotiana benthamiana* Domin (Gilroy *et al.* 2007) and *HvPap-1* in *H. vulgare* (Díaz-Mendoza *et al.* 2017). Recently, it has been reported that the reduced levels of *HvPap-1* and *HvPap-19* CysProt in barley alter leaf structures that confer differential susceptibility to biotic stresses mediated by pathogens and phytophagous species (Gómez-Sánchez *et al.* 2019).

4.2.1. Infestation by *Tetranychus urticae*

The two-spotted spider mite, *Tetranychus urticae* Koch, London strain (Acari: Tetranychidae), is one of the most polyphagous herbivores, considered as a damaging pest worldwide with a broad range of host plants, including barley (Santamaría *et al.* 2012a,b; Bensoussan *et al.* 2016). The interaction between phytophagous arthropods and plants generates HAMPs and DAMPs that are perceived by receptors on the plant cell surface and trigger intracellular signal transduction pathways that activate defence mechanisms (Santamaría *et al.* 2012a, b, 2013, 2018a,b; Rioja *et al.* 2017). The spider mite introduces its stylet across the epidermis between contiguous pavement cells or through the open stomata, to suck the content of leaf mesophyll cells (Fig. 3). Then, the plant will detect not only cell damage, but also herbivore elicitors such as salivary secretions. Even, salivary proteins can suppress SA-induced defenses (Jonckheere *et al.* 2016; Villaroel *et al.* 2016; Iida *et al.* 2019). Egg deposition and associated fluids can also be perceived as elicitors.

The analysis of *T. urticae* genome revealed outnumbered gut C1A and C13 CysProt as compared with other arthropods (Grbic *et al.* 2011). Also SerProt and Aspartic proteases have an important representation. These proteases can be targeted by PhyCys to be used as biotechnological tools for pest control. Arabidopsis plants overexpressing barley HvCPI-6 cystatin and BTI-CMe trypsin inhibitor were more efficient to confer leaf protection against spider mite damage than the single expression (Santamaría *et al.* 2012a).

4.2.2. Infection by *Magnaporthe oryzae*

Fungus pathogens are one of the greatest causes of crop losses (Godfray *et al.* 2016). Depending on their lifestyle, fungus plant pathogens are classified as biotrophs, necrotrophs or hemibiotrophs (Vleeshouwers and Oliver, 2014). The term of biotrophy, refers to microorganisms growing and colonizing live host plants, like *Blumeria graminis* f. sp. *hordei* on barley (Bindschedler *et al.* 2009). In the case of necrotrophic pathogens, the colonization and nutrition of the fungus takes place on dead or dying plant tissues. An example is *Rhynchosporium secalis* in barley (Sayed and Baum 2018). Hemibiotrophic pathogens are described as a mixture between both above mentioned. First, these fungi initiate a host plant interaction with living tissues, causing their deterioration and death. At this point, the fungi

can start the colonization of the dead tissues. This strategy is followed by *Magnaporthe oryzae*, commonly known as rice blast fungus (Fernandez and Orth, 2018). This organism can be found under different names, like *Pyricularia oryzae*, *Pyricularia grisea* or *Magnaporthe grisea*, depending on the sexual stage of their lifecycle (Ebbolle, 2007). This is a major threat to cultivated rice worldwide crops (Talbot *et al.* 2009), but also can infect other cereals, like barley (Zellerhoff *et al.* 2008; Tanaka *et al.* 2010; Ulferts *et al.* 2015). When a three-celled asexual spore (also called conidia) lands on the leaf surface, it germinates extruding the appressorium, a specific infection structure (Tucker *et al.* 2004; Talbot *et al.* 2009). The appressorium development depends on the spore subdivision phases.

When this structure reaches the mature stage, *M. oryzae* can either generate up to 8 MPa of turgor pressure to break the leaf cuticle or release enzymes to hydrolyze the cuticle and plant cell wall, generating a special structure known as penetration peg (Wilson and Talbot, 2009; Fernandez and Orth, 2018). To avoid losing turgor, mature appressorium deposits a thick layer of melanin on the inner side of the cell wall. This coat adds an impermeable barrier which prevents solute flow (Ryder and Talbot 2015). Wax monomers of plant cuticles are powerful inducers of appressorium development on the leaf surface (Ryder and Talbot 2015). The appressorium establishment is an essential stage during *M. oryzae* infection since it is the first structure that interacts with the plant (Fig. 4). This interaction takes place in the apoplast, in which plasma membrane acts as a pathogen-effector molecules and activate extracellular defense signaling pathway (van der Hoorn and Jones 2004; van der Hoorn 2018). B-like C1A CysProt are secreted to the apoplast and become active to protect plant host cells via activation of the HR, as occurs in *N. benthamiana* Domin plants. Hence, these proteases could be considered positive regulators for plant-fungus interaction (Gilroy *et al.* 2007).

Fig. 3. Cellular and molecular aspects of plant-*T. urticae* interactions. A) Summary schematics depicting potential determinants of plant-*T. urticae* interactions. The two-spotted spider mite feeds from the content of leaf mesophyll cells (dark and light green: mesophyll). Stylet (red thin line) crosses the epidermal leaf layer (in light blue) through the stomatal opening. The disrupted cuticle is shown by a red line. **B)** Composite of the scanning electron microscopy (SEM) image of spider mite feeding on a bean leaf and cross-section of a leaf, capturing feeding site with damaged and consumed cells (red asterisk) and part of the spider mite stylet (red arrowhead). The stylet penetrated the leaf epidermis through the stomatal opening. Adapted from Rioja *et al.* 2017.

Fig. 4. Schematic representation of appressorium development in the rice blast fungus *Magnaporthe oryzae*. The fungus three-celled conidium adheres to the plant hydrophobic cuticle and germinates (adhesion stage). One daughter nucleus migrates into the immature appressorium and a septum is formed, while the other nucleus migrates back to the conidium, which then goes through an autophagic cell death process. The appressorium remains mitotically active after maturation. Brown circles, nucleus; broken lines, conidium degradation. (Adapted from Fernandez and Orth 2018).

OBJECTIVES

Protein breakdown and mobilization are some of the major metabolic features associated with different aspects of the plant physiology, required for effective nutrient recycling and essential for the plant survival. Plant proteolysis includes a network of molecular events, subcellular compartments and different protease types and regulators, being C1A CysProt some of the most abundant key players. However, it is limited the number of members functionally described and the physiological processes where are involved. This PhD thesis tries to expand our knowledge on the barley-C1A CysProt system as a promising model to identify what proteases are involved in the plant response to drought in this species and which ones participate in the grain germination, to then, make a selection of this candidates for a further characterization. For this, the following objectives are proposed:

1. To exam the transcriptional response of the whole barley C1A CysProt family in leaves stressed by water limitation, as well as in barley grains upon germination. This study aims to select relevant members with significant expression to further investigations.
2. To molecularly and biochemically characterize the selected candidates, to analyze their phenotypes and to describe their proteolytic function using transgenic barley plants.
3. To decipher the effects of the candidate genes in the loss-of-function lines to determine their impact on the plant growth and behavior under drought conditions, and during germination.

MATERIAL AND METHODS

1. Plant material and growth conditions

Plants of barley (*H. vulgare* cv Golden Promise) were used for all experiments throughout this Thesis.

Barley grains were imbibed in distilled water for 5 min and then sterilized with 5% (v/v) sodium hypochlorite during 10 min under continuous shaking; then, they were rinsed with distilled water twice. Next, the grains were submerged in 5% (v/v) hydrochloric acid for 5 min followed by several washes with distilled water. 20 grains were placed onto a 150 mm Petri dish with moistened sterile double filter paper and left in darkness in a Sanyo MLR-350-H chamber at 22°C. The sterilized barley grains were germinated in individual pots (8 × 8 × 8 cm) with a mixture of soil and vermiculite (3/1), at 22°C under a 16/8 h light/dark photoperiod for 7 days in Sanyo MLR-350-H chambers. The plants, either subjected to treatments or controls, were grown in a greenhouse under the same conditions.

1.1. Drought treatment

Barley plants were deprived of water for 14 days. The controls were watered individually in alternate days. The pots were placed over plastic plates to individualize watering and maintain soil moisture at 70%, following Velasco-Arroyo *et al.* (2018a). Soil humidity was monitored by a sensor (SM150 Delta-T-Devices, Cambridge, UK) daily. After the treatment, all leaves were harvested, frozen into liquid nitrogen and stored at -80°C.

1.2. Generation of transgenic barley plants

Knock down lines for the C1A CysProt *HvPap-1* and *HvPap-19* genes, KD Pap1 and KD Pap19, respectively, were produced by the artificial microRNA (amiRNA) technology developed in the MicroRNA Designer Web platform (WMD3, <http://wmd3.weigelworld.org>). The amiRNA constructs were engineered from the pNW55 vector replacing the 21 bases of the natural Osa-MIR528 miRNA by 21 bases to specifically silence the *HvPap-1* or *HvPap-19* genes (5' TTATGCGGCATTGATACCGGT 3' and 5' TTTATTAACGGGCACATCCA 3', respectively). The final products of 554 bp were cloned into the p6d35S binary vector (DNA-Cloning-Service, Hamburg, Germany) using the pUbi-AB vector as intermediary. This binary vector included

the *HPT* (hygromycin phosphotransferase) selectable marker gene driven by the doubled enhanced CaM35S-promoter. Immature embryos were transformed with the binary vectors using the *Agrobacterium tumefaciens* Conn, strain AGL1 as described in Hensel *et al.* (2008).

Homozygous transgenic plants were obtained by the double haploid technology in collaboration with the Plant Reproductive Biology Group at the IPK in Gatersleben (Germany), according to Hensel *et al.* (2009) and Marthe *et al.* (2015), and grown as described above. Antibiotic resistance gene as selection marker was confirmed by PCR using specific primer pairs (Hensel *et al.* 2008).

1.3. Characterization of transgenic plants

The characterization of KD Pap1 and KD Pap19 lines was performed according to Díaz-Mendoza *et al.* (2016a) and Gómez-Sánchez *et al.* (2019), respectively. Whole leaves from 7 day-old plants were harvested, frozen into liquid nitrogen and stored at -80°C . From each transform event, four lines for KD Pap1 and KD Pap19 plants were selected based on mRNA levels, transgene copy number and protein content as is described in further sections.

2. *T. urticae* growth conditions and infestation

A colony of spider mite *T. urticae* was provided by Dr. Miodrag Grbic (University of Western Ontario, London, Canada). This colony was reared on bean plants (*Phaseolus vulgaris* L., cv California Red Kidney, Stokes, Thorold, Ontario, Canada) and grown in a Sanyo MLR-350-H growth chamber at 25°C and 70% relative humidity under a 16/8 h light/dark photoperiod, for more than one hundred generations. Hereafter, this colony was transferred to barley plants where it was maintained under the same conditions for more than 30 generations to ensure host adaptation following Santamaría *et al.* (2018b).

Seven day-old barley plants were infested with 20 barley-adapted females of *T. urticae* per plant. Mites were growing during 7 days in a growth chamber (25°C , 70% relative humidity and 16/8 h light/dark photoperiod). Barley plants were confined in pots with plastic cylinders covered on top by nylon nets to avoid dispersion of mites. The same isolation system was applied to control plants. Barley leaf damage was measured with a scanner (HP Scanjet 5590 Digital Flatbed Scanner). Leaf damage was calculated at 7 days post infestation

as described Santamaría *et al.* (2018b). Measurements were expressed as mm² of affected tissue by using Adobe Photoshop CS (Adobe Systems, San Jose, CA) software. Also, the damaged area was determined by the naked eye with a grid and calculated according to Cazaux *et al.* (2014). Three independent experiments were performed for each sample following to Santamaría *et al.* (2018b).

3. *M. oryzae* growth conditions and infection

The *M. oryzae* pCAMBIA-Guy11 strain fused to GFP (Green Fluorescent Protein) was kindly donated by Dr. Ane Sesma (CBGP, UPM-INIA). This strain was cultured according to Talbot *et al.* (1993) and Galhano *et al.* (2017) using the complete medium media (CM) composed by:

- Glucose 10 g/L
- Peptone 2 g/L
- Yeast extract 1 g/L
- Casamino acids 1 g/L
- Trace elements 0.1 % (v/v)
- Vitamin supplement 0.1 % (v/v)
- NaNO₃ 6 g/L
- KCl 0.5 g/L
- MgSO₄ 0.5 g/L
- KH₂PO₄ 1.5 g/L, pH 6.5

Barley plants were infected by spray inoculation following Díaz-Mendoza *et al.* (2017). To stimulate fungi colonization, each plant was covered with a plastic a bag and incubated for 24 h at 19°C. Afterwards, the plants were grown without the bags at 20°C and 65% relative humidity under a 16/8 h light/dark photoperiod. The symptoms were monitored at 3 and 7 days after infection. The leaves were scanned with a Scanjet 5590 Digital Flatbed Scanner HP. The damaged area was measured using the Assess 2.0: Image Analysis Software for Plant Disease Quantification (Amer Phytopathological Society), according to Sesma and Osbourn (2004). Expression levels of the *M. oryzae* small subunit of ribosomal RNA (Mo28S-rRNA) were quantified at 3 and 7 days post-inoculation (dpi).

4. Molecular biological methods

4.1. RNA Isolation

Barley tissues were crushed in a mortar and frozen in liquid nitrogen. RNA samples were extracted following the 8 M CILi method described by Oñate-Sánchez and Vicente-Carbajosa (2008). 0.2 g of barley tissue was resuspended in 550 µL of phenol + 550 µL extraction buffer (0.4 M LiCl, 0.2 M Tris HCL (pH 9), 25 mM EDTA and 1% SDS (w/v)) and vortexed twice. After two centrifugation steps, the supernatant was precipitated with 8 M CILi overnight. The pellet was washed with 200 µL 75% (v/v) ethanol and centrifuged again to eliminate the ethanol phase. The pellet was let to dry out, then resuspended in 25 µL of sterile water and frozen at -80°C. Once RNA was obtained, 2 µg of RNA were digested, using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions, to obtain the cDNA.

4.2. DNA extraction

Seven day-old frozen leaves were crushed in a mortar and 0.2 mg of the resultant powder were mixed with an extraction buffer (sarcosine 1 M, Tris HCL 100 mM pH 8, EDTA 10 mM, pH 8 and NaCl 100 mM) and vortexed. Then, a phenol:chloroform:isoamylalcohol solution (26:24:1) was added, mixed and centrifuged. The supernatant was transferred into a new tube containing a mixture of 3 M sodium acetate (pH 5,2) and isopropanol. After centrifugation, the pellet was washed out with isopropanol and resuspended in autoclaved water.

4.3. Quantification of nucleic acids

The concentration and purity of the RNA or DNA was verified by measuring the absorbance at 230, 260 and 280 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). To determine DNA quality, the 260/280 absorbance ratio was calculated. Values under 1.8 for DNA and 2.0 for RNA were considered not appropriate due to protein contamination.

4.4. Electrophoresis of nucleic acids

Agarose gels were prepared at different concentration (0.8-3 % (w/v)) depending on the nucleic acid sizes, according to methods described in Sambrook *et al.* (2006). Electrophoresis

was run in 1x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8) and 0.02% (v/v) ethidium bromide. The nucleic acid bands on the gels were visualized using a Gel Doc EZ Imager (Bio Rad) under UV light. GeneRuler DNA Ladder (Thermo Scientific) was used as marker of nucleic acid sizes.

4.5. Quantitative Real Time Polymerase Chain Reaction (RT-qPCR)

All the RT-qPCR analyses were performed by triplicate on a CFX96 Real-time system (BioRad) using the SYBR Green detection system. Quantification was standardized to barley cyclophilin (*HvCycl*) mRNA levels following Díaz-Mendoza *et al.* (2016a) and distilled water was used as a negative control were used to check possible contaminants. Table 1 contains the sequences of the primers used. The RT-qPCR conditions were: denaturalization in two steps (10 min followed by 15 s) at 95°C, annealing for 30 s at 60°C and extension for 20 s at 72°C, during 40 cycles. The melting curves were acquired between 65-95°C for 5s, then cooled down to 4°C.

GENES	PRIMERS
<i>HvCycl</i>	forward: 5'-CCTGTCGTGTCGTCGGTCTAAA-3' reverse: 5'-ACGCAGATCCAGCAGCCTAAAG-3'
<i>HvActin</i>	forward: 5'-GGTAGGGATGGGGCAGAAGG-3' reverse: 5'-ACCAGCGAGATCCAAACGAAGAA-3'
<i>HvPap-1</i>	forward: 5'-TCCTGGAGTCGATCTTTGGTTTC-3' reverse: 5'-CAAGCATACTGTTGCGGCTTC-3'
<i>HvPap-19</i>	forward: 5'-CACCTTATTCATGTCTGGCGAA-3' reverse: 5'-TGCCCGCTTAATTTGACAGG-3'
<i>Mo28S-rRNA</i>	forward: 5'-TACGAGAGGAACCGCTCATTGAGATAATT-3' reverse: 5'-TCAGCAGATCGTAACGATAAAGCTACTC-3'
<i>TuRp49</i>	forward: 5'-CTTCAAGCGGCATCAGAGC-3' reverse: 5'-CGCATCTGACCCTTGAAGTTC-3'

Table 1. Primers used for RT-qPCR assays. *HvCycl* (barley cyclophilin), *HvActin* (barley actin) *HvPap-1* and *HvPap-19* (barley CysProt), *Mo28S-rRNA* (*M. oryzae* small subunit of ribosomal RNA) and *TuRp49* (*T. urticae* ribosomal protein 49).

4.6. Gene copy number

Gene copy number of *HvPap-1* and *HvPap-19* in transgenic lines was calculated according to Díaz-Mendoza *et al.* (2016a) and Gómez-Sánchez *et al.* (2019), applying the $2^{-\Delta\Delta C_t}$ method reported in Falk *et al.* (2002) and Burton *et al.* (2004). The cyclophilin (*HvCycl*), the Osa-MIR528 miRNA (*MiR*) and 4-hydroxyphenyl-pyruvate dioxygenase (*Hv4Hppd*) genes were used as housekeeping calibrator genes; autoclaved water was employed as a negative control. Table 2 contains the sequences of the primers used.

GENES	PRIMERS
<i>HvCycl</i>	forward: 5'-CCTGTCGTGTCGTCGGTCTAAA-3' reverse: 5'-ACGCAGATCCAGCAGCCTAAAG-3'
<i>Hv4hppd</i>	forward: 5'-GCTCCAAATCTTCACCAAGC-3' reverse: 5'-CTCTTCCCCTCTCTCGTCCT-3'
<i>MiR</i>	forward: 5'-AGTTATGCGGCATTGATACCGGTCAGGAGATTGAGTTTGA-3' reverse: 5'-AATTATGCGGCATAGATTCCGGTAGAGAGGCCAAAAGTGAA-3'
<i>HvPap-1</i>	forward: 5'-TCCTGGAGTCGATCTTTGGTTTC-3' reverse: 5'-CAAGCATACTGTTGCGGCTTC-3'
<i>HvPap-19</i>	forward: 5'-CACCTTATTCATGTCTGGCGAA-3' reverse: 5'-TGCCCCGCTTAATTTGACAGG-3'

Table 2. Primers used for analyzing transgene copy number. *HvCycl* (barley cyclophilin), *Hv4Hppd* (barley 4-hydroxyphenyl-pyruvate dioxygenase), *MiR* (Osa-MIR528 miRNA gene) and *HvPap-1* and *HvPap-19* (barley CysProt).

FastStart Universal SYBR Green Master (Rox, Roche) was used for the quantification of double stranded DNA in a final volume of 20 μ L. The RT-qPCR reaction was performed in multiplates (Bio-Rad MLL9601) using PCR sealers TM (Bio-Rad MSB1001). The reactions were carried out in a C1000TM thermal cycler with CFX96TM optical reaction module and the results were analyzed with the CFX Manager Software 2.0 (Bio-Rad). The RT-qPCR conditions were the same as in 3.4.5.

5. Analysis of physiological parameters

5.1. Natural phenotype

Knock down (KD) lines (KD Pap1 and KD Pap19) and WT plants were grown in 32 cm diameter pots in the greenhouse until grain production, as described in 3.1. Their phenotypes were monitored and photographed with an OLYMPUS VR-320 digital camera every week at the same day and time throughout the life cycle.

5.2. Plant and soil water content

A soil moisture and temperature sensor (SM150 Delta-T-Devices, Cambridge, UK) was used to measure soil humidity every day as an indicator of drought stress. The aerial plant biomass was weighted in a balance (Precisa XB 2200 C) at the end of each drought treatment (fresh weight) and after drying in an oven at 70°C for 4 days (dry weight). Plant water content (PWC) was expressed as the percentage of the difference between fresh and dry weight. At least 6 plants of KD Pap1, KD Pap19 and WT lines were used.

5.3. Quantum yield efficiency

The efficiency of photosystem II was measured in the oldest leaf of 14 days old control and drought stressed KD Pap1, KD Pap19 and WT plants using a portable fluorometer (FluorPen FP 100, Photon Systems Instruments, Drasov, Czech Republic). Previously, the plants were kept in continued darkness for 20 min as described by Zmienko *et al.* (2015). Three independent biological replicates were measured.

5.4. Stomatal conductance

Stomatal conductance (gs) was measured by the vapor flux of control and 14 day-old drought treated plants. To calculate this parameter, the oldest leaf was introduced in a leaf porometer (SC-1 Decagon-T, Pullman, USA) for the . This experiment was realized with three independent biological replicates.

5.5. Germination rate, root and coleoptile lengths

Twenty grains of KD Pap1, KD Pap19 lines and WT plants were placed onto sterilized 150 mm Petri dishes with moistened sterile filter paper, following Díaz-Mendoza *et al.* (2016a). 12, 24, 36, 48, 60 and 72 hours after imbibition (hai) stages were measured. These developmental stages were registered with an OLYMPUS VR-320 digital camera and a stereomicroscope Leica MZ10 F equipped with a DFC 420 C Charge-Coupled Device (CCD) camera. The length of the emerging roots and coleoptiles was quantified in 72 hai grains using the program Fiji. Three independent replicates were made.

5.6. Grain weight, length and area

The weight, length and area of KD and WT barley grains were measured. The length and area of 100 grains per line were analyzed on a digital seed analyzer Marvin (GTA Sensorik GmbH device, MARVITECH, Germany). All the grains were distributed on the measuring surface, then photographed with a digital camera. The images were processed by the MARVIN software and the results were displayed in a log table. The weight of 100 grains per line was measured with a precision balance.

6. Biochemical analysis

6.1. Pigment content

Chlorophyll *a* and *b*, total chlorophyll and carotenoids (xanthophyll and carotenes) were quantified in control and drought-treated plants of KD lines and WT plants. 100 mg of leaves were ground in a mortar with liquid nitrogen and the resultant powder was resuspended in 15 ml of 80% (v/v) acetone in photo-protected tubes. After centrifugation at 13,000 *g* for 2 min, the absorbance of 1 ml of the supernatant was measured at 470, 663 and 646 nm, for carotenoids, chlorophyll *a* and chlorophyll *b*, respectively, using a UV-vis spectrophotometer (UltroSpec™ 3300pro, Amersham Bioscience). The pigment content was calculated using the extinction coefficient according to Lichtenthaler (1987). The results were expressed as mg/g of fresh weight.

6.2. Protein extraction and quantification

Total soluble proteins were extracted from treated and control leaves according to Díaz-Mendoza *et al.* (2016a). 0.2 g of leaf tissues were ground in liquid nitrogen. Then, 500 µl of extraction buffer (150 mM NaCl, 50 mM sodium phosphate, pH 6 and 2 mM EDTA) was added. After centrifugation at 16,300 *g* for 15 min at 4°C, the supernatant was recovered for protein quantification according to Bradford (1976), using bovine serum albumin (BSA) as standard. The protein content of barley deembryoned grains was measured using the same protocol.

6.3. Fractionation of albumins, globulins and hordeins from barley grains

Twenty grains after 0, 24, 72 and 96 hai were deembryonated, crushed in a mortar and resuspended with distilled water. Then were stirred continuously for 12 h at 4°C. After centrifugation at 5,900 *g* for 30 min at 4°C, the albumins were recovered from the supernatant and the globulins were extracted from the pellet by adding 5% (w/v) NaCl in distilled water, and then maintained at 4°C and centrifuged at 5,900 *g* for 30 min to recover the soluble fraction enriched in globulins. This protocol was adapted from Shi and Xu (2009). Hordeins were obtained from a parallel set of 20 crushed deembryonated grains at the same time points, and extracted after incubation in a buffer containing 55% (v/v) 2-propanol and 1% (v/v) 2-mercaptoethanol, for 1h at 60°C and after centrifuge step for 10 min at 13,300 *g* (Martínez *et al.* 2009).

6.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were separated in SDS-polyacrylamide gels (SDS-PAGE). The composition of the gels was: 15 % (v/v) acrylamide, Tris HCl 1.5M pH 8.8, 10% (v/v) SDS, 50% (v/v) glycerol, 10% (w/v) ammonium persulfate (APS) and 5 µL TEMED. Stacking gels were made by 4% acrylamide, Tris HCl 0.5M pH 6.8, 10% (v/v) SDS, 50% (v/v) glycerol, 10% (w/v) APS and 10 µL TEMED, following Laemmli (1970).

All the proteins were previously denatured by a heat treatment at 90°C for 5 min in a denaturalization buffer (12.5 mM Tris-HCl pH 6.8, 0.25% (w/v) SDS, 2.5% (v/v) β-

mercaptoethanol, 0.01% (w/v) bromophenol blue and 3.75% (v/v) glycerol). After this step, samples were run at 120 V during 90 min, and then stained with a Coomassie solution (50% (v/v) methanol, 0.05 % (v/v) Coomassie brilliant blue R-250, 10% (v/v) acetic acid and 39.95% H₂O) for 10 min. To wash out the excess of stain, gels were submerged into a destaining solution (50% methanol (v/v), 10% acetic acid (v/v) and 40% H₂O) for 1 h, and then kept o/n in a low distaining solution (5% (v/v) methanol, 7 % (v/v) acetic acid and 88% H₂O) at room temperature under continuous shaking. All gels were loaded with a molecular weight standard (Spectra™ Multicolor Broad Range Protein Ladder, Thermofisher).

6.5. Immuno-blot assays

Plant protein extracts were obtained from frozen KD and WT 7 day-old barley leaves. After denaturalization and separation in SDS-PAGE polyacrylamide gels, the proteins were transferred onto nitrocellulose membrane (GE Healthcare) during 40 min at 80 V, in a transfer buffer (48 mM Tris, 39 mM Glycine, 0.037 % (v/v) SDS, 5% (v/v) methanol). After blotting, the proteins were visualized by a Ponceau stain solution (0.5 % (w/v), 1% (v/v) glacial acetic acid). After H₂O washings and blocking with 3% (w/v) BSA diluted in PBS: Tween 20 0.05% (v/v) buffer for 1 h, a rabbit anti-*HvPap-19* antibody raised against the peptide NH₂-CQEKKHFSIDAYQVNSDPHD-COOH (Pineda Antibody Services, Berlin, Germany) was applied at a dilution 1:5,000 (v/v) in a buffer (0.05 % (w/v) skimmed milk, 0.1% (v/v) 10X PBS and 0.001 % Tween 20) and incubated overnight at 4°C. Afterwards, the membranes were washed with PBS 6 times and a secondary antibody anti-rabbit IgG conjugated to peroxidase (Sigma) was applied at a dilution 1:10,000 (v/v). For the detection of proteins, ECL Plus™ (GE Healthcare) reagent was used.

6.6. Protease activities

Proteolytic activities of barley deembryonated dry grains and grains at 24 and 72 hai ,were measured by hydrolysis of substrates containing the AMC (7-amino-4-methyl coumarin) fluorophore. Cathepsin B-like and L-/F-like activities were determined using Z-RR-AMC (*N*-carbobenzoxy-Arg-Arg-AMC) and Z-FR-AMC (*N*-carbobenzoxy-Phe-Arg-AMC) substrates, respectively, in a buffer containing 0.1 M sodium phosphate pH 6.5, 10 mM cysteine, 10 mM EDTA and 0.1% (v/v) Brij 35, at 30°C. 5 µg of protein extract and the corresponding substrate were added to a final concentration of 0.25 mM in a volume of 100 µL, according to Velasco-

Arroyo *et al.* (2018a). To detect trypsin-like activity the substrate Z-R-AMC (N-carbobenzoxy-Arg-AMC) and the protein extract were incubated substrate in a buffer containing 0.1 M Tris-HCl, pH 7.5, at 37°C for 1 h, following Arnaiz *et al.* (2018).

Fluorescence was measured with a 365-nm excitation and 465-nm emission filter (TECAN GENios™ Pro 96/384 Multifunction Microplate Fluorescence Reader). Triplicate assays were performed for determination of each value and the average was calculated. Commercial trypsin (EC 3.4.21.4), papain (EC 3.4.22.2), and bovine cathepsin B (EC 3.4.22.1) from Sigma, were used. Results were expressed as nmol of hydrolyzed substrate per mg of protein per min (nmol/mg*min). The system was calibrated with known amounts of AMC in a standard reaction mixture.

6.7. Amino acid quantification

Samples were obtained from leaves of plants that had been water-stressed for 7 days and from untreated controls of KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1776) and WT plants. Four replicates were used. 20 mg were resuspended in methanol, disrupted by three cycles of freeze/thawing and processed with a TissueLyser LT small bead mill (Qiagen). After centrifugation at 15,700 rpm for 20 min, the supernatant was transferred to a new tube and dried off in a SpeedVac. The metabolite extracts were resuspended by 1 min vortex mixing in 0.1 M of formic acid containing 0.2 M methionine sulfone (internal standard) and then centrifuged (15,700 rpm, 15 min). The clear solution was analyzed by capillary electrophoresis–electrospray ionization–time-of-flight MS (CE-ESI-TOF MS). Every sample was prepared and analyzed with three technical repeats.

Nineteen nitrogen metabolites (Ala, Arg, Asn, Asp, betaine, Ile+Leu, Gln, Glu, His, Lys, Met, Phe, Pro, sarcosine, Ser, Trp, Tyr, and Val) were analyzed by CE-ESI-TOF MS essentially as described by Moraes *et al.* (2011). The metabolites were previously identified in WT barley samples by comparison of their migration times and spectra with pure standards. A Capillary Electrophoresis System (7100, Agilent Technologies) coupled to a 6224 time-of-flight MS system (Agilent Technologies) was used. The separation occurred in a fused-silica capillary (Agilent; total length 100 cm, i.d. 50 µm). Separation was under normal polarity with a background electrolyte containing 1.0 mM of formic acid in 10% (v/v) methanol at 20°C. The sheath liquid (6 µl min⁻¹) was methanol/water (1/1, v/v) containing 1.0 mM formic acid with two reference masses to allow correction and thus higher accuracy in the MS. Samples were

hydrodynamically injected at 50 mbar for 35 s and stacked by injecting the background electrolyte at 100 mbar for 10 s. The optimized MS parameters were: fragmentor 150 V, skimmer 65 V, octopole 750 V, nebulizer pressure 10 psi, drying gas temperature 200°C, and flow rate 10.0 l min⁻¹. The capillary voltage was 3500 V.

Data were acquired in positive Dual-ESI mode with a full scan from *m/z* 87 to 1000 at a rate of 1.41 scan s⁻¹. The resulting CE-MS data files were cleaned of background noise and unrelated ions by the Targeted Feature Extraction tool with Profinder software (B.08.00, Agilent). Linear regression coefficients were calculated for each compound and metabolites were quantified in the samples.

6.8. Hormonal Analysis

Freeze-dried leaf material was ground to fine powder, weighed in 2-ml micro tubes and spiked with 25 µl of an internal standard mixture (containing ABA-*d*6, DHJA and ¹³C₆-SA) to correct for analytic losses. Extraction was carried out in 1 ml ultrapure water for 10 min in a ball mill at room temperature using 2 mm glass beads. After extraction, the homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C to remove the debris. Supernatants were recovered, the pH was adjusted to 3.0 with 30% (v/v) acetic acid, and partitioned twice against an equal volume of di-ethyl ether. The combined organic layers were evaporated under vacuum in a centrifuge concentrator (Jouan, Saint Germaine Cedex, and France). The dry residues were subsequently reconstituted in 0.5 ml of 10% (v/v) aqueous methanol and the resulting solutions filtered through 0.20-µm PTFE syringe membrane filters. Filtered extracts were analyzed by tandem LC/MS in an Acquity SDS UPLC system (Waters Corp., USA) coupled to a TQS triple quadrupole mass spectrometer (Micromass Ltd., UK) through an electrospray ionization source. Separations were carried out on a C18 column (Gravity C18, 50 × 2.1 mm, 1.8 µm particle size, Macherey-Nagel, Germany) using a linear gradient of ultrapure methanol and water, both supplemented with acetic acid to a 0.1% (v/v) concentration, at a constant flow rate of 0.3 ml min⁻¹. During analyses, the column was maintained at 40°C and the samples at 10°C to slow down degradation. Plant hormones were detected in negative electrospray mode by their specific precursor-to-product ion transitions (ABA, 263/153; JA, 209/59; SA, 137/93; SAGE, 299/93; OPDA, 291/165; JA-Ile, 322/130) and quantified using external calibration curves with standards of known amounts.

6.9. Starch and sugars content

Four grains of each line were ground in mortar and the Multi-Purpose FT-NIR Spectrometer Analyzer (Bruker™) was used to determine total starch and sugars content. Five biological replicates were introduced into FT-NIR vials for measurement after normalization of the residual moisture in a dry environment. The conditions of the measurements of the spectral regions of interest were developed according to OPUSLAB Validation Program (OVP) spectroscopy software standards. Glucose, fructose, sucrose, hexoses, starch and total of carbohydrates were obtained based on spectral regions of OH and CH stretching.

6.10. Total lipid content

Twelve grains were weighted using an electronic balance (ML54, Mettler-Toledo, Greifensee, Switzerland). Then, Time-domain NMR spectroscopy was performed on a Bruker 750 MHz Advance system (MQ60 instrument; Bruker GmbH, Rheinstetten, Germany) using a 5 mm birdcage resonator (Bruker), according to Borisjuk *et al.* (2005). Intact dry seeds were loaded in NMR tubes at 20 °C, and the spin echo signal acquired at 7 ms was averaged over 12 measurements. The signal height of the spin echo is proportional to lipid content, and the proportionality constant was estimated from a range of samples of known lipid content following Borisjuk *et al.* (2011).

Low-field TD-NMR application for high-throughput measurements was performed using a minispec mq60 (Bruker BioSpin GmbH, Rheinstetten, Germany). The NMR instrument has proton frequency of 60 MHz (1.4 T). The probe head allowed the analysis of samples with a diameter up to 6 mm and a length of 10 mm. The automated sample delivery platform was designed for a fully automated weighing of vials and vial transfer toward the mq60 NMR instrument. It consisted of a xyz-picker arm, an electronic balance and two sample racks of 250 vials each. The vials had to be filled manually with appropriate amounts of seed material, while all subsequent steps were programmed using the software and run fully automated. The software for robot operation was provided by Comicon GmbH (Hamburg, Germany).

7. Histochemical staining techniques

7.1. Lugol stain

Dry grains of KD and WT barley grains were imbibed in double-distilled water for 24 h. Then, they were longitudinally dissected and starch was stained with 20% (v/v) Lugol (Sigma). The results were visualized with a Leica MZ10 F stereo microscope and photographed with a Leica DFC420 C CCD camera, according to Díaz-Mendoza *et al.* (2016a).

7.2. Auramine O stain

Old leaves from drought-treated and control KD and WT plants were cut off in 1 mm pieces along the major axis. Every piece was put separately in a well of a 96 plate transparent plate and covered with water during 5 minutes. Leaf cuticles were stained with Auramine O to solution (Auramine O 0.01% (w/v), Tris-HCl 0.05 M, pH 7.2) during 5 min in darkness, according to Buda *et al.* (2009) Then, the samples were rinsed with distilled water, mounted with glycerol:PBS (1/1, v/v) on slides and observed on a Leica TCS SP8 confocal microscope. 12 replicates of each sample were used.

8. Immunohistochemistry and microscopy

8.1. Fixation and sectioning

WT 24 hai grains were fixed in a slight vacuum during 4 h at room temperature with 4% (v/v) formaldehyde and 0.5 % (v/v) glutaraldehyde in 50 mM PBS pH 7.0. Afterwards, the samples were washed twice with 50 mM PBS pH 7.0, for 15 min each. Samples were dehydrated with increasing concentrations of ethanol diluted in distilled water 30% and 50% during 30 min, 70% overnight, 90% for 30 min and 100% (v/v) twice for 1h at room temperature. Samples were infiltrated in 25% (v/v) of Roti®-Histol diluted in ethanol o/n, followed by two infiltration steps of 50% and 75% (v/v) of Roti®-Histol for 1 h each one and two steps of 100% of Roti®-Histol during 30 min each. The samples were infiltrated with Paraplast Plus (Sherwood Medical, St Louis, MO, USA) in increasing solutions (25%, 50%, 75% and 100% (v/v)) of Roti®-Histol. Finally, the specimens were embedding in 100% Paraplast Plus using

base moulds and cassettes, in a Leica EG1150 embedding station according to Borisjuk *et al.* (2013). 8 μ M sections were obtained in a rotary microtome Leica RM 2165 and mounted on glass slides.

8.2. Immunodetection

The sections on slides were submerged on 100% Roti[®]-Histol for 10 min twice, then they were rehydrated in a series of decreasing concentration of ethanol in PBS (100% twice, 90%, 70%, 50% and 30%, for 2 min each) and PBS for 2 min twice. To block non-specific protein, the samples were incubated with SEABLOCK buffer (ThermoFisher[®]) for 120 min, following Madureira *et al.* (2006). Afterwards, the samples were incubated with the rabbit primary antibody (either anti-HvPap-1 or anti-HvPap-19) at a 1/50 dilution in AB buffer (BSA 1 % (w/v), 0.005 % Triton X-100 and TBST, pH 8.4) for 60 min. After 5 washes of 5 min with washing buffer (BSA 0.1 % (w/v), 0.005 % Triton X-100 and TBST, pH 8.4), the secondary antibody anti-rabbit conjugated was applied at a dilution of 1/250 in AB buffer for 60 min. Subsequently, followed by 5 washes for 5 min with washing buffer and treated with DIG detection buffer (Roche[®]) for 10 min. Afterwards, the sections were incubated with the Vector detection system (Impact[™] Vector[®] Red, Vector Laboratories) during 12 min to develop the signal and finally rinsed with Millipore water three times. The specimens were mounted in VectaMount and observed on a ZEISS Axioplan-2 under bright field and photographed with a CCD colour Leica DFC 300FX, 1,4 MPixels camera.

PROTEASES	ANTIBODY-RECOGNIZED PEPTIDES
HvPap-1	SGFAPSRFKEKPYWIIKN
HvPap-19	CQEKKHFSIDAYQVNSDPHD

Table 3. CysProt amino acid sequences used for specific antibody production.

8.3. Confocal microscopy

Auramine O staining of leaf cuticle was detected using a Leica TCS SP8 confocal microscope under the excitation with the Argon laser line of 458 nm and the emission of the dye was collected in the range 490–560 nm. Stacks of xyz and xzy series were obtained and then visualized using the free software Leica LAS X (<https://www.leica-microsystems.com/products/microscope-software/details/product/leica-las-x-ls/>) and Fiji (<https://imagej.net/Fiji/Downloads>). For the xzy series, xz planes (perpendicular to the leaf surface) were taken along the y-axis. The distance between the first and the last xz planes was 8.12 μm for all xzy series. Up to 50 individual measurements were taken along the cuticle of the maximum projections of the xzy images using the program Fiji. The stomatal apertures were contoured and their area measured using Fiji.

KD and WT leaves infected with the fungus *M. oryzae* were monitored at two time-points, 3 and 7 days after infection, using the Leica TCS SP8 confocal microscope. The growth of the fungus was detected by the expression of green fluorescent protein (GFP, excitation 488 nm, emission range 494–564 nm). Chlorophyll auto fluorescence was used as a marker of cell viability (excitation 633 nm, emission range 699–741 nm). Random tacks of 21 xyz planes were collected at 40X magnification along the apical 2 cm of the leaves.

9. Statistical Analysis

Normality and homogeneity of variances were tested at first, using for it Shapiro-Wilk test and Levene test, respectively.

For the experiments in which were analyzed the comparison of protease expression in control and drought conditions, and to compare the effects of the genotype under control or drought treatments, was employed Student's t-test.

One-way-Anova was used to compare the effects of the genotype under control or drought conditions, cuticle thickness, stomatal pore area, metabolite contents, bioassays (comparison foliar damage and pathogen/pest) and physiological parameters. For this arrangement of experiments, one-way-ANOVA was followed by post-hoc Student-Newman-Keuls (SNK) multiple comparison tests.

Also was used for realize grain phenotype (length, width, area and weight), sugar, oil and protein content, enzymatic activity, and coleoptile and root length. In this bulk of experiments, the post-hoc choice based on number of replicates and homogeneity variance was Tukey's (honestly significant difference, HSD) multiple comparison test.

To compare the accumulation of hormones and the number of leaves, two-way-ANOVA was employed, followed a SNK multiple comparison tests.

In all of cases, P-values less or equal to 0.05 were considered statistically significant. The software Graph Pad Prism 6 was used for all statistical analyses.

RESULTS

1. Response of barley C1A CysProt genes to drought

The expression profile of the 42 C1A CysProt genes in barley leaves after 14 d of water deprivation was assessed by RT-qPCR assays. mRNA expression levels normalized to the constitutive active cyclophilin gene revealed that 12 barley cathepsin genes were expressed under control and/or drought conditions. Among these 12 cathepsins, two B-like, one F-like, the only one H-like, and 8 out of the 34 L-like cathepsins were found. However, only four protease gene members, *HvPap-8* (cathepsin L-like), *HvPap12* (cathepsin H-like), *HvPap1* (cathepsin F-like) and the cathepsins B-like and *HvPap-19*, were significantly induced in stressed leaves in comparison to control plants (Fig. 5).

Fig. 5. Expression of CysProt genes in barley leaves under drought and control conditions. All 42 C1A CysProt genes in barley were assessed by RT-qPCR after 14 d of drought or watering. Only the relative mRNA levels of those that were expressed under drought and/or control conditions are shown. Values were normalized using barley cyclophilin gene. Data are the mean ($\pm$ SE) of three independent biological replicates. Significant differences between means were determined using unpaired t-tests: * $P < 0.05$.

The implication of barley CysProt in drought stress was further examined using knock-down plants. Aside from the KD Pap1 lines (1128, 1130, 1175 and 1176) previously characterized by Díaz-Mendoza *et al.* (2016a), transgenic plants for the *HvPap-19* gene (KD Pap19 lines) were generated using the same artificial amiRNA approach via *Agrobacterium*-mediated

transformation. Homozygous KD Pap19 lines were selected for further characterization based on transgene copy number, and transcript and protein levels. Following these criteria, the KD Pap19 lines 1770, 1776, 1779 and 1782 were chosen (Supplementary Fig. S1). Lines 1770 and 1776 contained a single copy of the transgene whereas lines 1779 and 1782 had two gene insertions, estimated by RT-qPCR assays and the $2^{-\Delta\Delta Ct}$ method. mRNA expression levels in all samples were reduced as compared to the control. Immunoblot assays using a specific antibody against a peptide of HvPap-19, already checked to avoid cross-reactivity (Díaz-Mendoza *et al.*, 2016a), showed a reduction in the accumulation of the protein content in comparison with the WT (Supplementary Fig. S1). However, neither mRNA nor protein content was completely knocked out, confirming the knock down character of these lines.

Fig. 6. Expression levels of *HvPap-1* and *HvPap-19* genes in leaves of different barley genotypes under drought and control conditions. Expression of *HvPap-1* and *HvPap-19* genes in knock down KD Pap1 (1128, 1130, 1175, 1176 lines), KD Pap19 (1770, 1776, 1779, 1782 lines) and WT plants after drought stress and watering (control) conditions. Data were determined by RT-qPCR and expressed as relative mRNA levels, normalized to *cyclophilin* mRNA content.

Since *HvPap-1* and *HvPap-19* mRNAs were up-regulated after drought stress in WT plants, the expression patterns in KD lines for both genes were likewise studied after water deprivation. In comparison to WT plants, a significant reduction of both *HvPap-1* and *HvPap-19* gene expression was observed not only in the corresponding KD line but also in the KD line for the other transgene, either grown under watered or drought-stressed conditions. However, the mRNA levels of *HvPap-1* gene in drought-stressed KD Pap19 lines were higher than that reached in the KD Pap1 lines. In contrast, mRNA levels of *HvPap-19* gene were similar in KD Pap1 and KD Pap19 lines before and after drought stress (Fig. 6).

2. Barley KD Pap1 and KD Pap19 lines show phenotypic structural alterations

The phenotypes of the three barley genotypes were compared after 14 d of water deprivation vs watering conditions. WT and KD Pap19 watered-plants looked more robust and tended to have higher size than KD Pap1 plants. Under water stress, WT plants seemed more affected and showed less leaf turgor, while the leaves of both KD lines stayed upright. In addition, leaf growth of KD Pap19 lines tended to be greater than KD Pap1 leaves (Fig. 7).

Fig. 7. Barley phenotypes after drought treatment. Phenotypes of WT, KD Pap1(1130 and 1175 lines) and KD Pap19 (1770 and 1776 lines) barley plants after 14 d of water deprivation (drought) or watering (control).

The whole growth cycle of KD Pap1 and KD Pap19 plants was compared to WT plants. No relevant phenotypic alterations were observed between transgenic and WT plants during the first 8 weeks of development. By the end of their lifespan, when the spikes appeared, a slight delay in natural senescence was observed in the *HvPap-1* amiRNA lines in contrast to the non-transformed plants. Senescence delay in KD Pap1 lines has been previously reported by Díaz-Mendoza *et al.* (2016a). In contrast, KD Pap19 phenotypes were quite similar to the WT plants through the complete life cycle (Fig. 8).

Fig. 8. Phenotype of barley plants at 4, 6, 8, 10 and 12 weeks of development. WT, KD Pap1 (1128, 1130, 1175 and 1176 lines) and KD Pap19 (1770, 1776, 1779 and 1782 lines) plants. Pictures are representative of three independent experiments grown during the same period of the year.

Interestingly, leaves from transgenic and WT plants showed apparent differences in surface texture and hardness. To evaluate these potential appreciations, further analysis on leaf cuticle structure and thickness were determined by microscopy confocal after an Auramine-O staining procedure. Regarding the cuticle on the adaxial side of the leaf, the thickness of the leaves increased significantly only in the transgenic KD Pap1 lines grown under control conditions (Fig. 9). Under water deprivation, leaf cuticles become thicker in the three barley genotypes. In particular, KD Pap1 lines presented significant thicker cuticles than KD Pap19 and WT plants. Moreover, the outer shape of epidermal cells of water-stressed leaves seemed more irregular than the controls (Fig. 9). Although the average number stomata per unit of leaf surface area was not modified either among lines or in stressed vs non-stressed leaves (data not shown), stomata changes were found in position, size and aperture as can be observed under microscope. Generally, the stomata tended to be located in a lower position comparing to the general epidermal cells, particularly in transgenic leaves after water deficit (Fig. 9).

Based on these differences observed among the three barley genotypes, the stomata pore was measured. Results showed that the stomata pore area of watered-WT leaves was larger than in the transgenic leaves under same conditions. While leaves of WT and KD Pap1 plants maintained their stomata open under watered conditions, in KD Pap19 lines stomata were almost closed (Fig. 10). Under drought stress, the stomata pore area of KD Pap19 lines was larger than those from KD Pap1 and much larger than in WT plants, which correlated with the stomata aperture stage (Fig. 10).

Fig. 9. Cuticles of barley leaf epidermis stained with Auramine O. (A-J) Fixed width (8.12 μm) stacks of xz planes across the y-axis were collected from leaves using a confocal microscope. Upper labels show the water treatment (control or drought); and plant genotypes are indicated as follows in the side labels: KD Pap1 (lines 1130, 1175), KD Pap19 (lines 1770, 1776) and WT plants. Asterisks indicate the positions of stomata in the epidermal layers. (K) Images were treated with Fiji software to quantify the cuticle thickness. This quantification was performed by taking up to 50 measurements per image. Values are means ($\pm\text{SE}$) of measurements from three independent biological replicates. Different letters indicate significant differences as determined using one-way ANOVA followed by a SNK test ($P < 0.05$). Significant differences between pairs of means were determined using unpaired *t*-tests. * $P < 0.05$.

Fig. 10. Stomata of leaf epidermis stained with Auramine O. Leaves come from water deprivation and control of KD Pap1 (1130 and 1175 lines), KD Pap19 (1770 and 1776 lines) and WT plants. Stacks of xyz series (parallel to the leaf surface) collected on a confocal microscope (A-J). The stomata areas were manually contoured and measured using the program Fiji. Results represent the mean $\pm$ SE of at least twelve replicates from three independent biological experiments. Different letters indicate significant differences ($P < 0.05$, One-Way ANOVA followed by Student-Newman-Keuls test).

To characterize the potential interplay between cuticle/stomata features under drought stress in barley KD lines and WT plants, some physiological parameters associated to abiotic stresses were analysed. Soil water content was severely reduced after 14 d of drought treatment, independently of the barley genotype (Supplementary Fig. S2A). Dry and fresh weight did not show any difference among barley genotypes when the plants were grown under a water deficit regime (Supplementary Fig. S2B-C). An important reduction on other drought markers, such as total chlorophyll and carotenoid content was detected in stressed

vs control-plants but no relevant differences were found between transgenic and WT plants (Supplementary Fig. S2D,F). In contrast, a significantly higher quantum efficiency of photosystem II and an increase in total protein content were found in drought-stressed KD Pap1 and KD Pap19 lines in comparison to stressed WT, while no differences in both parameters were observed in all tested plants under watered conditions (Fig. 11).

Fig. 11. Physiological parameters of barley KD Pap1, KD Pap19, and WT plants after 14 d of drought or control treatments. (A) Quantum yield and (B) protein content. Data are means ($\pm$ SE) of at least six replicates from three independent biological experiments. Different letters indicate significant differences as determined using one-way ANOVA followed by Student-Newman-Keuls tests ($P < 0.05$).

3. Metabolomics changes are associated with drought stress in HvPap-1 and HvPap-19 knock down lines and WT plants

To verify whether the differential protein accumulation detected in leaves was related to differences in the free amino acid composition, a metabolomics analysis was performed by comparing the amount of free amino acids and related compounds, in transgenic and WT leaves after drought or watering regimes. Significant differences between the concentration of numerous amino acids were found between the knock down lines and the WT, both under control and drought conditions (Supplemental Table S1.). In KD Pap1 lines, most compounds had a significant higher or lower concentration value than in WT plants. In spite of these quantitative differences, the contribution of each compound to the total set shared quite similar trends in all lines, even in the more divergent KD Pap1 1130 line (Fig. 12). The amino acids Glu, Gln, Asp and Asn, and the compounds betaine and sarcosine were the most abundant metabolites in watered conditions. The contribution to the total pool of metabolites decreased for these compounds in all lines after drought treatment, with the exception of betaine, which even increased. A strong increase of Pro in the total pool was found together with minor rises of several hydrophobic amino acids such as Leu + Ile, Val, Tyr and Trp after water deprivation.

Fig. 12. Heatmap of the composition in free amino acids and related compounds before and after 14 d of drought in barley leaves of KD Pap1, KD Pap19, and WT plants compared with control conditions. WT, KD Pap1 (1130 and 1175) and KD Pap19 (1770 and 1776) lines were analyzed by control (C) and drought (D) conditions. The color-coding shows the level of abundance quantified as the proportion (percentage of total) of each metabolite in the different samples. The original values have been $\ln(x+1)$ -transformed. No scaling is applied to the rows. Both rows and columns are clustered using correlation distance and average linkage.

4. Differential accumulation of hormones among KD and WT lines in response to drought

The content of some hormones was determined in plant leaves grown under water deprivation or watered conditions to analyse whether the barley responses to drought stress were equally regulated among the three barley genotypes (Fig. 13 and Table 5).

Fig. 13. Quantification of hormones in barley KD Pap1, KD Pap19 and WT plants after 14 d of drought or control treatments. Analysis from lines 1130, 1175 (KD Pap1) and 1770, 1776 (KD Pap19). (A) OPDA, 12-oxo-phytodienoic acid; (B) JA, jasmonic acid; (C) JA-Ile: JA-isoleucine; (D) ABA, abscisic acid; SA, (E) salicylic acid; (F) SAGE, salicylic acid glucosyl ester.

Results demonstrated that OPDA levels, the precursor of JA pathway, were higher in leaves of KD Pap1 lines grown under control conditions and that their levels decreased reaching similar values among all the tested samples after drought treatment.

However, the JA and its bioactive form JA-Ile, were dramatically accumulated in response to drought in KD Pap1 and WT leaves. Although a homogeneous pattern was not observed for JA and JA-Ile in watered KD Pap19 lines, their levels were significantly lower than those of WT and KD Pap1 after drought stress (Fig. 13A-C). As expected, the ABA concentration was greatly increased under water deficit in all leaf samples but the amount of ABA was significant higher in both stressed KD lines than in WT plants (Fig. 13D). Finally, the amount of SA was not altered either among lines or between treatments and its glycosylated form, the Salicylic Acid Glucosyl Ester (SAGE) only showed significant differences in KD Pap19 lines, with higher levels after drought treatment (Fig. 13E,F).

5. Knock down *HvPap-1* and *HvPap-19* lines differentially respond to biotic stresses

Since leaf cuticle and stomata were differentially altered in KD Pap1 and KD Pap19 lines compared to WT plants, the effect of these structural changes was analysed in the defence response to pathogens and pests. This was performed by comparing transgenic and WT plants after the fungal infection mediated by *M. oryzae* and the infection mediated by the phytophagous acari *T. uticae*.

Initially, the fungus *M. oryzae* infection produced spots with necrotic borders that may enlarge and coalesce to colonize the entire leaf (Fig. 14). The highest fungus injury was detected in leaves from KD Pap19 lines, particularly in the oldest leaf (L1) and in the apex of the leaf 2 (L2), being the damaged area extremely large at 7 d of infection. In contrast, the damaged leaf area of KD Pap1 lines was significantly smaller. In infected WT leaves, an intermediate damage was found at both time points, 3 and 7 d (Fig. 14).

Fig. 14. Plant phenotypes and leaf damage after *M. oryzae* infection of leaves of barley KD Pap1, KD Pap19, and WT plants. Plant phenotypes after (A) 3 d and (B) 7 d of infection. (C) Quantification of leaf damage measured as mm² of injured foliar area in all leaves of each plant after 3 d and 7 d of infection. Data are means ($\pm$ SE) from four independent plants. Different letters indicate significant differences as determined by one-way ANOVA followed by Student–Newman–Keuls tests ($P < 0.05$).

To discern the cellular events taking place during the early barley-fungus interaction, fluorescent GFP-labelled *Magnaporthe* isolates were used to inoculate barley plants. Confocal fluorescence microscopy revealed that the fungus invasive hyphae was highly developed in KD Pap19 lines which inversely correlated with the leaf autofluorescence due to the chlorophyll, a marker of cell viability. This result confirms differences in leaf damage (Fig. 15D,E). An intermedium equilibrium between fungus-GFP and chlorophyll-fluorescence was detected in infected WT leaves while autofluorescence was more prominent in infected KD Pap1 lines (Fig. 15A-C).

Fig. 15. Confocal images of leaves of barley KD Pap1, KD Pap19, and WT plants infected with *M. oryzae* expressing the protein pCAMB-Guy11 fused to green fluorescent protein. The images were taken at 3 d post-inoculation. The green fluorescence of the hyphae and chlorophyll autofluorescence, as a marker of tissue damage, was detected using a confocal microscope. Series of 21 xzy images were collected from random areas along the apical 2-cm of the leaves. Maximum projections are shown.

To study the impact of the *HvPap-1* and *HvPap-19* genes on the *M. oryzae* performance, RT-qPCR assays were carried out to measure the mRNA levels corresponding to the small subunit of ribosomal RNA (*Mo28S-rRNA*). Results showed a significantly higher quantity of fungus mRNA in leaves of KD Pap19 in comparison to KD Pap1 and WT plants at 3 d of infection (Fig. 16). After 7 d, the low fungus mRNA levels found in the KD Pap19 lines was probably due to the death of most leaf tissue in these lines.

Fig. 16. Expression of a *M. oryzae* constitutive gene after infecting different barley genotypes. Effects of the three barley genotypes (KD Pap1, KD Pap19 and WT plants) on *M. oryzae* performance at 3 and 7 d after fungus infection. Quantification of fungus small subunit of ribosomal RNA (*Mo28S-rRNA*) mRNA expression levels. Results represent the mean $\pm$ SE of three independent biological replicates. Different letters indicate significant differences ($P < 0.05$, One-Way ANOVA followed by SNK test).

In addition, mite bioassays were performed on transgenic and WT plants and their phenotypes were monitored after 7 d of feeding. Small chlorotic spots appeared due to the mite stylet penetration and plant cell sucking. These symptoms were evident at 7 d of mite

feeding (Fig. 17), but much more injury was observed at longer infestation time (data not show).

Fig. 17. Plant phenotypes and mite performance after 7 d of infestation with *T. urticae* for barley KD Pap1, KD Pap19 and WT plants. (A) Quantification of leaf damage measured as mm² of injured foliar area in all leaves of each plant. (B) Total number of leaves per plant of mite-infested and non-infested plants. (C) Mite performance as quantified by the relative expression levels of the *T. urticae* ribosomal protein 49 (TuRp49) as determined by RT-qPCR. Data are expressed as mRNA levels normalized to barley *cyclophilin*. All data are means ($\pm$ SE) of either (A, B) four plants or (C) three independent biological replicates. Different letters indicate significant differences as determined by either (A, C) one-way or (B) two-way ANOVA followed by Student-Newman-Keuls tests ($P < 0.05$). (D) Representative examples of mite-infested leaves, where L1 is the oldest leaf and L2 is the next leaf on the plant.

However, infested KD Pap1 lines showed less leaf damage than infested WT plants and the number of leaves per plant was not modified in comparison to non-infested plants. In contrast, the mite impact on KD Pap19 lines was severe since the leaf injury was much higher than in infested WT. Moreover, the number of leaves per plant was reduced in KD Pap19 lines under mite infection (Fig. 17A,B). *T. urticae* performance was also monitored after feeding on the all barley lines. In order to do that, mRNA levels of a mite progress gene marker, Ribosomal Protein 49 (*TuRP49*), were quantified. A significant increment of *TuRP49* gene expression was found when mites fed on KD Pap19 lines which correlated with the major leaf damage. The low *TuRP49* mRNA levels were associated to the little leaf damage observed in KD Pap1. Intermedium mite gene expression levels and damaged foliar area were detected in infested WT plants when compared to KD lines (Fig. 17C).

6. *HvPap-1* and *HvPap-19* participate in barley grain development and germination

Previous data had reported the expression of different C1A CysProt genes, including *HvPap-1* and *HvPap-19*, in barley grains (Martínez *et al.* 2003; Sreenivasulu *et al.* 2008; Díaz-Mendoza *et al.* 2016a). A search in a microarray analysis performed with different tissues of barley grain at different time points during grain development and germination, revealed that both genes, *HvPap-1* and *HvPap-19*, were expressed in both processes (unpublished results, Dr. V. Radchuk, IPK-Gatersleben, Germany). These results were validated in this work by RT-qPCR assays using specific primers (Table 1), and referred to the normalized mRNA levels of the constitutive *actin* gene (Fig. 18). The two genes were continuously expressed in different barley tissues during grain development since the very early stages (initiation of flowering) until the last stages tested (24 daf) (Fig. 18A,B). Likewise, transcripts of *HvPap-1* and *HvPap-19* genes were accumulated in the embryo and endosperm of the mature grain up to the first 36 hai, although the expression levels of the *HvPap-19* gene were much higher than those from the *HvPap-1* gene. In particular, *HvPap-19* was specifically high in 24 hai endosperm (Fig. 18C).

To correlate *HvPap-1* and *HvPap-19* gene expression during grain germination with the localization of *HvPap-1* and *HvPap-19* proteases, immuno-detection assays were performed

in barley grains sections at 24 hai using antibodies against specific peptides derived from the HvPap-1 and HvPap-19 proteases (Díaz-Mendoza *et al.* 2016a). The results showed a clear link between transcripts and protein accumulation patterns. The HvPap-1 protein was restricted to the aleurone cell layers, pericarp and vascular bundles (Fig. 19C,F,H,J) whereas the HvPap-19 protein was also accumulated in endosperm besides aleurone layers, pericarp and vascular bundles (Fig. 19D,G,I,K). The highest quantities of HvPap-19 protein were found in the aleurone layers and particularly in the pericarp. No signal above background levels was detected using rabbit pre-immune serum as negative control (Fig. 19B,E).

Fig. 18. Expression of *HvPap-1* and *HvPap-19* genes throughout grain development and germination. Data were determined by RT-qPCR and expressed as relative normalized mRNA levels to barley *actin* gene. Developing grains (DG) were sampled every 2 days since 0 to 24 daf. Endosperm (E) and embryo (Em) were sampled at 6, 12, 24 and 36 hai. The data are mean $\pm$ SE of three independent biological replicates. Different letters indicate significant differences ($P < 0.05$).

Fig. 19. Location patterns of HvPap-1 and HvPap-19 proteins in germinating barley grains by immunodetection. (A) Structure of longitudinal dissected barley grain. Cross sections of barley grains at 24 hai corresponding to the former endosperm transfer cells area (B,C,D), the basal grain area (E,F,G,H,I) and vascular bundle area (J,K) are shown. Antibodies used are indicated in Table 3. A: aleurone; GC: grain coat; E: endosperm; Em: embryo; P: pericarp; T: testa; VB: vascular bundle.

7. Barley grains of silencing *HvPap-1* and *HvPap-19* lines and WT are phenotypically and different

Knock down barley lines for *HvPap-1* and *HvPap-19* genes (KD Pap1 and KD Pap19, respectively) generated by amiRNA technology (Díaz-Mendoza *et al.* 2016a; Gómez-Sánchez *et al.* 2019) were used to study the influence of the altered expression of these two genes on the grain composition and on the germination process. Kernels from transgenic and WT lines were compared by measuring some physical and biochemical parameters. No relevant morphological differences or alterations in the mature grain size were observed among genotypes, after measuring the grain area, length and width, with just some exceptions in any of the parameters (Supplementary Fig. 3A,B,C). However, KD Pap1 and KD Pap19 plants produced grains with less weight than WT grains, and this reduction was particularly significant in the case of the KD Pap1 lines (Supplementary Fig. 3D). These differences in weight correlated with changes in the grain composition.

The starch content, as the major component of the mature grain, was significantly reduced in the grains of KD Pap1 lines in comparison with the starch accumulated in grains of WT and KD Pap19 lines (Fig. 20A). The dark blue intensity after Lugol staining corroborated these results reflecting higher levels of starch in WT grains than in transgenic lines (Supplementary Fig. 3). In contrast, the accumulation of sucrose was lower in grains of KD Pap19 lines than in grains of WT and KD Pap1 lines (Fig. 20B). Regarding the carbohydrate monomers, minor variations on the fructose content were found among the three barley genotypes, and the alterations detected on the glucose concentration appeared to be dependent on each plant instead of on the genotype (Fig. 20 C,D). Quantification of the total carbohydrates and lipids indicated that KD Pap1 grains contained less sugars than KD Pap19 and WT plants (Fig. 20E), but no significant changes in the accumulation of lipids were observed (Fig. 20F). Notably, the total protein content was different among the three genotypes. Dry grains from both transgenic lines presented significantly higher protein quantities than the non-transformed WT grains (Fig. 20G).

Fig. 20. Grain composition of barley transgenic lines and WT plants. Content of starch (A), sucrose (B), fructose (C), glucose (D), total carbohydrates (E) and total lipids (F). Values, expressed as percentage, are referred to grams per 100 grams of fresh weight and represent the means of five independent replicates. (G) Total protein content is expressed as μg of total protein per grain. KD Pap1 (1130 and 1175 lines), KD Pap19 (1770 and 1776 lines) and WT plants. Different letters indicate significant differences ($P < 0.05$).

8. Germination is delayed in transgenic KD Pap1 and KD Pap19 lines

To analyse whether the germination was somehow affected by the changes in grain composition, kernels from transgenic and WT plants were imbibed in watered paper on Petri dishes, and germination rates were monitored every 12 h for the first 72 hai. Considering that the germination was produced when the protrusion of the radicle tip through the seed coat took place (Bove *et al.* 2001), the first germinated grains were detected at 24 hai. Nevertheless, some grains, particularly those of WT plants, germinated before 24 hai. Germination of WT grains reached 100% within 48 hai while the germination rates found in most of the transgenic grains were lower, even at longer time points (Fig. 21). A significant delay in grain germination was observed for both KD lines, mainly at 24 hai, which was maintained during the process until the 72 hai. Most of the KD Pap1 grains started to germinate at 24 hai and gradually achieved the maximum (near 80-100%) at 72 hai while the germination percentages for KD Pap19 grains were around 60-90 range and many of them did not reach higher values. Among grains within the same genotype, some slight variations were also observed being particularly evident in the KD Pap19 lines.

The germination delay found in all tested transgenic grains was also observed in the growth pattern of roots and coleoptiles derived from the corresponding grains. Roots and coleoptile lengths from transgenic grains were significantly shorter than those from WT plants at 72 hai, and needed longer time to reach same lengths and sizes than WT plants (Fig. 22).

Fig. 21. Germination process in barley transgenic and WT plants. (A) Cumulative germination percentages of grains during the first 72 hai. (A) Percentage of germinated grains of KD Pap1 (1128, 1130, 1175 and 1176 lines), KD Pap19 (1770, 1776, 1779 and 1782 lines) and WT. (B) Images of the germination process in transgenic and WT grains at 24 and 72 hai of 1130, 1175 (KD Pap1) and 1770, 1776 (KD Pap19) lines.

Fig. 22. Quantification of root and coleoptile lengths at 72hai. KD Pap1 (1128, 1130, 1175 and 1176 lines), KD Pap19 (1770, 1776, 1779 and 1782 lines) and WT. Different letters indicate significant differences between lines ($P < 0.05$).

9. Grain protein content is altered upon germination in transgenic barley lines in comparison to WT grains

Since the total quantity of proteins was much lower in WT dry grains than in both transgenic lines (Fig. 20G) and the germination rates inversely correlated with these values (Fig. 21), a depth analysis on the protein content in the grain was performed. The quantity of the main storage proteins, hordeins, albumins and globulins, in the barley grains were determined at different germination time points in WT and in some selected transgenic lines. As expected, the total quantity of proteins decreased during germination with some differences among

the three genotypes (Fig. 23A), due to their degradation to be used as nutrient source for the embryo growth and the new plant development. In the fractions enriched in hordeins, the most abundant prolamins accumulated in the barley grain, the hordein values expressed as μg of protein/grain were higher in the transgenic dry grains than in the WT ones, with the exception of line 1776. These higher levels were maintained up to the 96 hai tested, particularly in KD Pap1 grains (Fig. 23B). The content of albumins in dry grains was not significant different between the WT and the transgenic lines, but after imbibition a significant higher quantity of albumins was quantified in KD Pap1 grains at the three time points analysed. KD Pap19 grains also presented higher albumin content at 72 and 96 hai in comparison to WT germinating grains (Fig. 23C). In the case of globulins, imbibed grains of both transgenic KD lines also showed higher accumulation levels than the WT imbibed grains (Fig. 23 D). In summary, the rate of remaining proteins was higher in grains of transgenic lines than in WT grains. The different dynamic profile of grain protein reserves among genotypes was corroborated by electrophoretic band patterns performed at different hai (Supplementary Fig. 4).

Fig. 23. Quantification of protein content in WT and transgenic barley grains at different hai. (A) Total grain protein. (B) Hordeins. (C) Albumins. (D) Globulins. KD Pap1 (1130 and 1175 lines), KD Pap19 (1770 and 1776 lines) and WT. Different letters or symbols indicate significant differences between lines for each time point ($P < 0.05$). 1130, 1175 (KD Pap1) and 1770, 1776 (KD Pap19) lines were used. Capital, small, Greek letters and Greek symbols are used for dry grain, 24, 72 and 96 hai grains, respectively.

10. CysProt and SerProt differentially participate in WT and transgenic barley grain germination

Differential germination rates between WT and transgenic grains could be due to variations in the sugar and protein composition of the grain but also can be a direct consequence of the action of the hydrolases responsible of the mobilization of the grain reserves. According to Sreenivasulu *et al.* (2008) and Díaz-Mendoza *et al.* (2016a), CysProt and SerProt are the main proteases involved in the barley grain germination. Thus, cathepsin L-/F- and B-like CysProt and trypsin-like SerProt were analysed in whole grains of both WT and transgenic lines at different germination times. The three protease activities were detected in all samples analysed (Fig. 24). No significant differences in cathepsin L-/F-like activities were observed between WT and transgenic either in dry or imbibed grains (Fig. 24A). In contrast, a sharp significant increase of cathepsin B-like activity was shown in KD Pap19 grains at 72 hai in comparison to WT and KD Pap1 lines (Fig. 24B). Besides, all transgenic lines showed a significant increase of trypsin-like activity at 24 hai being reduced at 72 hai while the temporal profile of trypsin activity in WT was the opposite, higher levels at 72 hai than at 24 hai (Fig. 24C).

In addition, embryos detached from transgenic and WT grains at different germination time points were used to determine the temporal expression patterns of *HvPap-1* and *HvPap-19* genes by RT-qPCR assays, instead of using whole or de-embryonated grains since the quality, and quantity of their RNAs was not good enough to perform an accurate analysis. Transcripts of both genes were gradually reduced as germination proceeded in WT embryos, being the mRNA levels almost undetectable at 96 hai (Fig. 25). The expression profile of the *HvPap-1* gene in KD Pap1 lines presented in general lower levels than in WT lines and KD Pap19 lines, except at 96 hai. A similar expression pattern was observed for the *HvPap-19* gene being the mRNA content lower in both KD lines at 24 and 72 hai and slightly increased at 96 hai, although the relative expression values were very low.

Fig. 24. Proteolytic activities in whole grains of WT and transgenic lines. (A) Cathepsin L-/F-like CysProt activity. (B) Cathepsin B-like CysProt activity. (C) Trypsin-like SerProt activity. KD Pap1 (1130 and 1175 lines), KD Pap19 (1770 and 1776 lines) and WT. Different letters indicate significant differences between lines for each time point ($P < 0.05$, HSD). Information about 1130, 1175 (KD Pap1) and 1770, 1776 (KD Pap19) lines. Capital, small and Greek letters are used for dry grain, 24 and 72 hai grains, respectively.

Fig. 25. RT-qPCR analyses of the mRNA accumulation in embryos of WT and transgenic lines at 24, 72 and 96 hai. *HvPap-1* and *HvPap-19* CysProt genes were analysed and 1130, 1175 (KD Pap1) and 1770, 1776 (KD Pap19) lines were employed. Relative expression level was normalized to barley cyclophilin mRNA content. The data are the mean $\pm$ SE of 8 replicates. Different letters indicate significant differences between lines within each time point. ($P < 0.05$ One-way ANOVA followed by Tukey's test)

GENERAL DISCUSSION

Protein breakdown and mobilization are some of the major metabolic features involved in many biological functions in plants, either associated with basic endogenous events during their lifespan or with the specific responses to external abiotic and biotic stresses. Among the more than 800 proteases encoded by plant genomes, C1A CysProt are key players in a variety of proteolytic mechanisms for plant survival, being particularly important in stress responses and seed/grain germination (Grudkowska and Zagdanska 2004; Martínez *et al.* 2012; Rawlings *et al.* 2018; Tornkvist *et al.* 2019). This thesis has focused in the barley-C1A CysProt system as a promising model to identify what C1A proteases act in the plant physiology under drought conditions as well as in the barley grain germination, to then, select candidates for deciphering the physiological mechanism behind these processes, and to finally bioengineer plants with favorable properties.

The increase in the mRNA levels of a gene is often related to an acquisition of the correct cellular state to ensure the best physiological condition. Based on this premise, we tested the expression levels of the whole CysProt family members in barley leaves after water deprivation. Besides, we searched the transcriptomic profile of CysProt genes in a microarray performed in barley grain upon germination. Among the differential expressed genes common in the two physiological processes, *HvPap-1* and *HvPap-19* encoding a cathepsin F- and B-like, respectively, presented the highest expression levels in both processes and were selected for a deeper functional analysis.

The success of plant survival under water deprivation, in the current context of global warming, depends on the ability to sense the stress and make adjustments to mitigate the impact. Plants induce molecular mechanisms, modify physiological processes and adapt their growth patterns to optimize an efficient use of water under drought stress (Osakabe *et al.* 2014). Altered CysProt expression are often linked to these changes since protein turnover with a rapid degradation and mobilization of proteins are enhanced under stress conditions (Kunert *et al.* 2015; Velasco-Arroyo *et al.* 2016). The analysis of expression levels of the 42 barley C1A CysProt after 14 d of water deficit revealed an induction of four of them, *HvPap-1*, *HvPap-8*, *HvPap-12* and *HvPap-19* genes, suggesting a putative role of these encoded CysProt in proteolytic processes specifically triggered by drought stress.

Most efforts to understand the role of CysProt under a water deprivation scenario have been made by overexpressing genes ectopically (Chen *et al.* 2010; 2013; Zang *et al.* 2010). In this work, for the first time, CysProt knock down lines have been generated to study plant responses to drought. The *in vivo* behaviour of transgenic *HvPap-1* and *HvPap-19* barley lines under a drought regime showed differences in some biochemical and physiological parameters associated with the stress in comparison to WT plants. While drought caused a significant decline in maximum quantum yield of photosystem II in WT plants, the efficiency of this photosystem was almost not altered in the knock down lines. Likewise, the total amount of proteins, an important parameter associated with drought, was highly reduced in stressed leaves of the WT but only slightly decreased in the two transgenic lines. These findings demonstrated the participation of these two CysProt in the plant adaptation to overcome this abiotic stress. The pattern of proteolytic activity in knock down lines did not show significant differences compared with the WT (data not shown), probably due to compensating effects as a consequence of the functional redundancy of CysProt that has been reported previously (Velasco-Arroyo *et al.* 2016; 2018a). Thus, although the contribution of each metabolite was quite similar in all lines both before and after water deprivation, drought induced a greater increase in many free amino acids in KD lines than in WT, consisted with proteolytic compensation effects. The increase in proline and betaine, two osmolytes with protective roles of subcellular structures in stressed plants, is known to be related to enhanced tolerance to drought (Ashraf and Foolad 2007). This increase in metabolites would protect the plants from dehydration.

As previously reported, barley KD Pap1 plants presented a slight delay in senescence compared to WT plants, particularly evident at the end of the natural senescence process (Velasco-Arroyo *et al.* 2016). So, adjustments in growth seemed to be mainly dependent on the CysProt gene. There are examples of plants expressing CysProt transgenes with different growth behaviour patterns. Transgenic Arabidopsis plants constitutively overexpressing *PSPC2* and *SPCP3* CysProt genes from sweet potato presented earlier floral transition from vegetative to reproductive growth, besides to alteration in seed germination in comparison to WT (Chen *et al.* 2010; 2013). On the other side, *HvPap-1* overexpressing barley lines exhibited a similar growth phenotype to that observed for WT plants (Velasco-Arroyo *et al.* 2016). Interestingly, the delayed senescence-phenotype of the KD Pap1 lines was accompanied by the presence of thicker leaf cuticles than WT plants, and alterations in the stomata position, size and aperture. These unexpected structural modifications, which were

even more notably after water deprivation, clearly supported the longer life cycle since cuticles acted as physical barriers that protect and help their survival (Bi *et al.* 2017; Xue *et al.* 2017). It is difficult to find the link between the low expression of a CysProt gene in the KD lines and the alteration in the structure of the leaf cuticle. CysProt participate in multiple physiological processes either directly involved in degrading proteins or sometimes processing pre-proteins to generate active forms, such as enzymes (Grudkowska and Zagdanska, 2004; Martínez *et al.* 2012). This led us to hypothesize that the different enzymes required for the synthesis of the cuticular wax components and cutin matrix could be somehow the target of the CysProt. Cutin and wax, components of the cuticle, are synthesized in the ER and exported via ATP-binding cassettes (ABC) transporters across the plasma membrane to the polysaccharide cell wall (Yeats and Rose 2013; Xue *et al.* 2017). Barley CysProt and cystatins have been localized in the ER and Golgi apparatus (Martínez *et al.* 2009; 2012); thus, an unbalance in the enzymes in the KD lines could affect the structure and/or composition of the cuticle. Alternatively, pleiotropic effects associated with the silencing of the CysProt genes due to a parallel reprogramming of other genes could have mediated the leaf structural changes that were observed.

Among the physical adaptations to drought stress, an efficient stomata structure is essential for an appropriate and rapid response. Usually, plant growth is impaired under water deficit due to a decrease in stomatal opening that limits CO₂ uptake and has negative consequences on the photosynthesis (Osakabe *et al.* 2014). Although stomata tend to be closed under water deprivation, leaves from KD Pap1 and especially from KD Pap19 plants kept their stomata open. This could be an adaptation to compensate the reduction in the transpiration process due to the presence of their thicker cuticles (Kosma *et al.* 2009; Bi *et al.* 2017). In addition, drought stress led to an increase accumulation of ABA, the main driver of stomata closure to diminish gas exchange and transpiration, and to reduce water loss (Osakabe *et al.* 2014; Sah *et al.* 2016). The high ABA concentrations detected in leaves of drought-stressed plants, and particularly in both KD lines, is in line with its role as modulator to avoid water losing. Since these high levels do not result in stomatal closure, the KD lines might be deficient in their ability to sense ABA. In parallel, the accumulation of JA and its active form JA-Ile in water-stressed KD Pap1 and WT plants was in agreement with data reported in other plant species grown under water deprivation (Mahouachi *et al.* 2007; De Ollas *et al.* 2015). Recent reports have demonstrated that JA regulates a subset of plant responses to drought by modulating ABA biosynthesis and accumulation (De Ollas and Dods 2016; De

Ollas *et al.* 2018). Curiously, JA and JA-Ile levels were significantly lower in leaves of water-stressed KD Pap19 lines than in KD Pap1 and WT plants. This hormone is potentially required for the maximum sensitivity of stomata to drying soil (De Ollas *et al.* 2018), and the JA and JA-Ile low levels could be correlated with the stomatal apertures observed in these plants even under water deficit regime.

Cuticles are not only barriers to desiccation but also are protective structures to biotic stress. On this bases, we challenged the different phenotypes (KD lines and WT plants) with the fungal pathogen *M. oryzae* and the phytophagous pest *T. urticae*. As expected, KD Pap1 plants, whose leaves presented thicker cuticles, resulted less damaged after 7 d of fungal infection or mite feeding than WT plants. In contrast, the foliar damage detected in KD Pap19 plants after the pathogen and the pest attack was higher than in WT plants. The leaf damage produced by the fungus or the mite correlated to the amount of fungus and mite mRNAs determined in each case. The enhanced defense shown by KD Pap1 lines against *T. urticae* attack was not in line with previous data from bioassays performed by Díaz-Mendoza *et al.* (2017). Probably, the different plant response was due to differences in the mite population used to infest plants. While Díaz-Mendoza *et al.* (2017) employed mite adults adapted to barley, here the infestation was performed with a barley-adapted synchronized population of female mites. Only females at the same stage of development were used for the feeding bioassays. At this point, the different susceptibility of KD Pap1 and KD Pap19 plants to biotic stresses probably did not only depend on the cuticle thickness. For example, hormonal content and regulators actively participate in the responses triggered by biotic stresses and act as important cues for producing greater resistance. Previous reports examining the effects of pathogens and insects on leaves either overexpressing or silencing CysProt have shown that the plant responses and the impact on the pathogen/pest are specific for each plant-organism interaction (Pechan *et al.* 2002; Gilroy *et al.* 2007; Shindo *et al.* 2012; Höwing *et al.* 2014).

We can conclude that barley *HvPap-1* and *HvPap-19* encoding cathepsin F- and B-like CysProt, respectively, are functional genes involved in drought stress processes. Their absence, even partial, alters phenotypic traits and unexpectedly produces leaf structural alteration that may confer differential susceptibility to abiotic and biotic stress.

Upon germination the role of plant proteases in the mobilization and of storage proteins accumulated in seeds and cereal grains has been largely established in both dicot and monocot species since a controlled proteolysis is crucial for the accurate provision of peptides and amino acids to sustain embryo growth until photosynthesis is completely established (Fisher *et al.* 2000; Tan-Wilson and Wilson 2012; Szewinska *et al.* 2016). In barley, members of the CysProt C1A family known as papain-like and C13 family termed legumain-like, have been described as the most important enzymes throughout the grain development and germination (Koehler and Ho 1988; 1990; Grudkowska and Zagdanska 2004; Sreenivasulu *et al.* 2008; Shi and Xu 2009; Martínez *et al.* 2009; Díaz-Mendoza *et al.* 2016a; 2019; Radchuk *et al.* 2018). Our search of *HvPap-1* and *HvPap-19* genes in the transcriptomic data derived from the microarray performed by Dr. Radchuk, and the subsequent validation, indicated that both genes were continuously expressed during the grain life, since the initiation until the last days of flowering, as well as in the grain tissues during germination. These expression patterns correlated with their protein immunolocations in the germinating grain. A barley grain represents a caryopsis in which the endosperm surrounded by the aleurone layer occupies the bulk of the grain and the pericarp is fused with the thin seed coat at maturity. Physiologically, CysProt are synthesized in the scutellar epithelium and aleurone layer and then secreted to the endosperm to degrade the stored prolamins to release peptides and amino acids for the embryo growth (Mikkonen *et al.* 1996; Martínez *et al.* 2009; Cambra *et al.* 2010; 2012b; Díaz-Mendoza *et al.* 2016a). The location of *HvPap-1* and *HvPap19* proteins predominantly detected in aleurone, pericarp and endosperm clearly coincides with these germination events suggesting that they may be major enzymes responsible for the proteolytic activity in these tissues.

To demonstrate the *in vivo* involvement of these two CysProt during germination, we generate knock down plants for both genes. The analysis of the grain morphology and composition in the transgenic lines showed that in the two knock down lines, the grain dry weight decreased and the grain content was altered. The total amount of proteins was higher in the transgenic than in control grains according to the prediction, which suggested that the reduction in the cathepsin F- and B-like activities limited the degradation of their protein targets. This is in agreement with the data reported by Díaz-Mendoza *et al.* (2016a) who found that most amino acids, considered end-products of proteolysis, were reduced in the mature grain when the *HvPap-1* was silenced. In addition, the increase of proteins in the transgenic endosperms seemed to be concomitant with a reduction of the carbohydrate

content. Grains of KD Pap1 and KD Pap19 plants accumulated less starch and sucrose, respectively, suggesting a specific adjustment of pathways to maintain appropriated levels of storage compounds in the endosperm. Amendments of metabolic pathways alter carbon/nitrogen fluxes and modify the accumulation of carbohydrates and proteins in the grain. In accordance to this hypothesis, Faix *et al.* (2012) reported that the reduction of starch synthesis in the endosperm of barley *Risϕ16*, deficient in cytosolic ADP-Glc pyrophosphorylase, did not stimulate the protein biosynthesis but increased the amount of free sugars and amino acids in the grain. Likewise, wheat grains overexpressing the sucrose transporter *HvSUT1* in the endosperm significantly increased grain protein content but also deregulated the metabolic status of grains, accompanied by an inverse oscillatory behaviour of regulators related to sugar signalling (Weichert *et al.* 2010). These differences in stored molecules imply important changes in the expression of genes and the corresponding synthesis of proteins as is shown by the novel techniques based on shotgun proteomics, activity-based protein profiling, and comparative and structural genomics that achieve a general view of the proteolytic process during germination (Díaz-Mendoza *et al.* 2019).

The increase of protein levels in the knock down barley grains specially affect the hordein accumulation which are the most abundant proteins stored in the starchy endosperm. HvPap-1 and HvPap-19 proteases have the capacity to particularly hydrolyze these hordeins generating peptides and amino acids (Martínez *et al.* 2009; Cambra *et al.* 2012b; Díaz-Mendoza *et al.* 2016a). Probably, they are also involved in the mobilization of albumins and globulins. The storage of different molecules that alter the grain composition and the ability to mobilize these compounds are directly linked to the germination process. KD Pap1 and KD Pap19 lines presented a delay in the germination. A decrease in the number of germinated grain was found in both knock down lines over time, in terms of developmental stage compared to WT. The reduction of HvPap-1 and HvPap-19 proteases inferred less cathepsin F- and B-like activities and consequently lower protein degradation which impaired the nutrient mobilization and a delayed the germination.

It was expected that alterations in the content of HvPap-1 and HvPap-19 triggered changes in the expression of genes encoding other proteases. Enzymatic activities measurements confirmed this hypothesis and highlighted the functional redundancy of protease isoforms and at the same time the enzymatic compensation between proteases of different classes such as cathepsins and trypsins as was previously described by Díaz-Mendoza *et al.* (2016a).

These changes are adaptation strategies developed by the plant to minimize the adverse effects of a limited or altered protein degradation in the grain to insure the correct release of nutrients and the accurate C/N uptake for the embryo growth until the onset of photosynthesis.

This is still an ongoing study and a relevant question to address is to decipher the correlation between the proteolytic activity upon germination and the alteration in the amino acid content in the grains of the three barley genotypes. It is expected that in the course of germination, the quantity and type of amino acid was altered and those related with N mobilization such as Gln and Asn presented major changes. Besides, it is of interest to know whether the alterations in the proteins and amino acids affect the lipid content, mainly stored in the embryo.

This research may have impact not only in the crop yield but also in food industry. Approximately, 75-80% of global barley production is used for animal feed, 20-25% for malting in brewing industry, 2-5% for human food, and the remaining part is used for the biofuel industry (Tricase *et al.* 2018). In the production of alcoholic beverages based on the fermentation, grains presenting high levels of carbohydrates would be wanted, as well as a faster germination rate. For livestock feeding industry, grains with a high level of proteins are desirable (Bleidere and Gaile, 2012). In conclusion, barley grains with a right proportion C/N depends on their uses and applications (Tricase *et al.* 2018). Finally, the potential of exploring the properties of plant proteases acting along seed germination will open new fields of research, as a biotechnological alternative to provide new prevention strategies protein-caused illnesses such as human gluten-related disorders (Martínez *et al.* 2019).

CONCLUSIONS

CONCLUSIONS

Among the 42 C1A peptidase genes identified in the barley genome, the *HvPap-1* and *HvPap-19* genes encoding a cathepsin F- and B-like, respectively, were selected for a deep functional analysis under the water limitation context and during the grain germination. The study allowed to conclude:

1. *HvPap-1* and *HvPap-19* genes were commonly up-regulated in water-stressed leaves and in germinating grains.
2. *HvPap-1* and *HvPap-19* knock down barley plants (KD Pap1 and KD Pap19) did not show relevant changes during their growth cycle in comparison to WT plants, but a slight delay in natural senescence at the end of the lifespan was observed in the KD Pap1 lines.
3. Morphologically, KD Pap1 and KD Pap19 plants had thicker cuticles and showed stomata changes in position, size and aperture in comparison to WT plants. These alterations were more remarkable under water deficit to overcome this stress. In fact, higher quantum efficiency of photosystem II and an increase in the total protein content besides differential changes in hormones were found in drought-stressed KD Pap1 and KD Pap19 lines in comparison to stressed WT.
4. The changes in the leaf cuticle thickness and stomatal pore area had advantageous effects on leaf defence against fungal infection and mite feeding mediated by *M. oryzae* and *T. urticae*, respectively.
5. *HvPap-1* and *HvPap-19* proteins were differentially immune-detected in germinating grains. While *HvPap-1* protein was restricted to the aleurone cell layers, pericarp and vascular bundles, *HvPap-19* protein was also accumulated in endosperm, suggesting a differential protein targets to be degraded upon germination.

6. KD Pap1 and KD Pap19 plants produced grains with less weight than WT grains, being this reduction particularly significant in the case of the KD Pap1 lines. These differences in weight correlated with changes in the grain composition.
7. Delayed germination phenotypes observed in KD Pap1 and KD Pap19 plants correlated with a higher protein content in the KD lines, mainly hordeins and globulins, and agreed with the role of these proteases in degrading grain-stored proteins.

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SUPPLEMENTAL FIGURES

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Fig. S1. Characterization and selection of transgenic lines. Selection of knock down HvPap-19 (KD Pap19) homozygous barley lines generated by amiRNA and double haploid technology. (A) Construct, number of events per construct, number of independent homozygous lines analyzed per transformation event and final selected lines used for molecular characterization. (B) Estimation of transgene copy number by RT-qPCR assays coupled to the 2- $\Delta\Delta C_t$ method. CN: copy number for each group. (C) Expression levels for HvPap-19 gene in KD Pap19 barley lines and WT plants analyzed by RT-qPCR technology, is referred as relative mRNA levels normalized to barley *cyclophilin* mRNA content. Data represent the mean $\pm$ SE of $n=6$ replicates from three independent experiments. (D) Western-blot of HvPap-19 protein in transgenic and WT plants using a specific peptide antibody. 37 and 29 KDa sizes correspond to unprocessed and processed protein forms, respectively (Martínez *et al.* 2012). Ponceau staining indicates protein charge control.

Fig. S2. Physiological parameters in different barley genotypes. Different parameters monitored in samples of KD Pap1, KD Pap19 and WT plants after 14 d of water deprivation (white bars) or grown under optimal watering regime (black bars). (A) Soil water content. (B) Fresh weight. (C) Dry weight. (D) Stomatal conductance. (E) Total chlorophyll content. (F) Total carotenoid content. Results represent the mean $\pm$ SE of at least six replicates from three independent biological experiments. Different letters indicate significant differences ($P < 0.05$, One-Way ANOVA followed by SNK test).

Fig. S3. Measures of physical parameters of WT and transgenic barley grains. Grain length (A), width (B), area (C) and weight (D) of WT, KD Pap1 (1128, 1130, 1175 and 1176 lines) and KD Pap19 (1770, 1776, 1779 and 1782 lines) grains. Different letters indicate significant differences. The data are mean $\pm$ SE of 73 replicates. ($P < 0.05$ One-way ANOVA followed by Tukey's test).

Fig. S4. Representative examples of the phenotypes and starch staining of WT and transgenic barley grains. (A) Structure of whole and longitudinal dissected grains 24 hai from WT and KD lines. (B) Lugol's iodine staining of WT and KD Pap1 (1130 and 1175 lines), KD Pap19 (1770 and 1776 lines) grains.

Fig. S5. Electrophoretic protein patterns of whole barley grains of WT and transgenic lines at different germination time points (0, 24, 72 and 96 hai). (A) Total protein. (B) Hordeins. (C) Albumins. (D) Globulins. WT and KD Pap1 (1130 and 1175 lines), KD Pap19 (1770 and 1776 lin

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	WT_C	1130_C	1175_C	1770_C	1776_C	WT_D	1130_D	1175_D	1770_D	1776_D
Ala	90.14 ± 0.74	126.47 ± 1.69 [*]	162.21 ± 4.61 [*]	94.00 ± 0.81	127.27 ± 1.27 [*]	311.60 ± 5.15	190.97 ± 2.56 [*]	514.34 ± 6.62 [*]	331.36 ± 15.26	332.57 ± 17.62
Arg	6.56 ± 0.05	14.95 ± 0.22 [*]	6.55 ± 0.08	6.41 ± 0.07	6.46 ± 0.02	10.04 ± 0.11	11.73 ± 0.04	18.59 ± 0.13 [*]	12.27 ± 0.38	11.94 ± 0.66
Asn	154.67 ± 1.70	870.45 ± 13.49 [*]	197.59 ± 2.73	143.64 ± 2.71	129.26 ± 0.22	232.98 ± 3.26	627.16 ± 3.17 [*]	131.64 ± 0.65 [*]	143.39 ± 8.83 [*]	232.12 ± 6.80
Asp	296.59 ± 1.91	227.31 ± 0.87 [*]	268.67 ± 3.68	271.21 ± 5.05 [*]	294.86 ± 2.46	112.82 ± 1.10	240.58 ± 2.71 [*]	63.49 ± 0.92 [*]	78.57 ± 0.89	99.45 ± 2.25
Betaine	436.93 ± 1.82	378.75 ± 2.88 [*]	467.89 ± 10.64	390.36 ± 5.19 [*]	519.77 ± 1.86 [*]	1859.66 ± 15.90	1440.66 ± 19.07 [*]	2173.49 ± 17.52	1672.01 ± 56.26	2113.76 ± 73.32
Gln	330.92 ± 2.98	745.60 ± 11.26 [*]	584.01 ± 5.03 [*]	458.82 ± 6.75 [*]	531.14 ± 4.82 [*]	477.83 ± 4.95	870.04 ± 5.95 [*]	672.79 ± 8.14 [*]	516.23 ± 19.31 [*]	531.24 ± 20.45
Glu	1491.41 ± 10.05	1142.01 ± 5.43 [*]	1367.64 ± 19.11 [*]	1277.56 ± 14.98 [*]	1406.02 ± 4.41	368.39 ± 4.41	675.58 ± 6.03 [*]	308.47 ± 2.17	280.66 ± 10.83 [*]	371.46 ± 12.30
His	4.01 ± 0.01	22.94 ± 0.27 [*]	3.84 ± 0.04	3.82 ± 0.03	3.33 ± 0.00	53.14 ± 0.29	26.68 ± 0.09 [*]	122.06 ± 1.30 [*]	81.54 ± 3.72 [*]	72.00 ± 3.65
Leu+Ile	41.85 ± 0.18	49.66 ± 0.68 [*]	46.88 ± 0.53 [*]	36.58 ± 0.49 [*]	37.31 ± 0.29 [*]	253.79 ± 1.77	134.77 ± 0.57 [*]	461.53 ± 1.50 [*]	337.50 ± 15.34 [*]	294.53 ± 13.70
Lys	5.27 ± 0.03	15.36 ± 0.28 [*]	5.41 ± 0.03	5.60 ± 0.07	5.60 ± 0.01	11.95 ± 0.11	14.16 ± 0.03	18.27 ± 0.12 [*]	16.67 ± 0.66	16.08 ± 0.82
Met	1.68 ± 0.02	1.64 ± 0.02	1.70 ± 0.02	1.68 ± 0.04	1.99 ± 0.01 [*]	2.09 ± 0.07	2.54 ± 0.04	3.73 ± 0.11 [*]	2.47 ± 0.06	2.78 ± 0.09
Phe	30.19 ± 0.23	28.78 ± 0.16	34.24 ± 0.68 [*]	30.42 ± 0.30	30.22 ± 0.17	81.40 ± 0.55	45.62 ± 0.51 [*]	154.24 ± 1.09 [*]	120.17 ± 4.25 [*]	99.24 ± 3.52
Pro	44.91 ± 0.33	61.54 ± 2.16 [*]	57.52 ± 4.10 [*]	48.57 ± 1.09	40.04 ± 0.21	4123.88 ± 62.35	1881.51 ± 12.84 [*]	5852.83 ± 70.64 [*]	4955.65 ± 241.66 [*]	4671.00 ± 263.46
Sarcosine	295.64 ± 2.13	357.14 ± 2.58 [*]	434.33 ± 7.90 [*]	325.95 ± 3.86	419.23 ± 3.26 [*]	419.79 ± 3.74	613.98 ± 2.03 [*]	382.80 ± 1.34 [*]	257.53 ± 12.19 [*]	397.79 ± 15.57
Ser	110.56 ± 0.75	155.94 ± 0.99 [*]	148.67 ± 2.51 [*]	115.99 ± 1.46	129.73 ± 0.50 [*]	117.85 ± 0.69	158.64 ± 0.64 [*]	82.05 ± 0.44 [*]	78.06 ± 2.81 [*]	96.70 ± 3.34
Trp	5.18 ± 0.05	5.52 ± 0.07	4.48 ± 0.07 [*]	4.33 ± 0.06 [*]	4.36 ± 0.03 [*]	60.82 ± 0.37	12.05 ± 0.12 [*]	158.32 ± 3.30 [*]	106.50 ± 4.60 [*]	76.42 ± 2.69
Tyr	16.69 ± 0.11	15.44 ± 0.12	18.04 ± 0.35 [*]	15.18 ± 0.18	15.55 ± 0.15	96.34 ± 0.65	37.06 ± 0.15 [*]	129.12 ± 3.61 [*]	117.52 ± 2.75 [*]	96.60 ± 3.33
Valine	80.09 ± 0.33	96.36 ± 1.19 [*]	104.68 ± 1.18 [*]	80.39 ± 0.93	78.07 ± 0.55	356.00 ± 2.16	207.18 ± 1.13 [*]	495.51 ± 4.70 [*]	423.34 ± 19.52	414.40 ± 17.32

Table S1. Amino acids and related compounds in barley leaves. Content of free amino acid and related compounds before and after 14 d of drought stress in leaves of KD Pap1 (1130 and 1175 lines), KD Pap19 (1770 and 1776 lines) and control (WT) plants. Data, referred as µg g⁻¹ dry weight are means ± standard error of six samples coming from two independent biological replicates. Red and green asterisks mark significant differences between each knock down line and its corresponding WT in control or drought). conditions (P<0.05, One-Way ANOVA followed by SNK test.

	WT_C	WT_D	1130_C	1130_D	1175_C	1175_D	1770_C	1770_D	1776_C	1776_D
OPDA	123.99 ± 57.39	33.18 ± 8.69	229.84 ± 31.35	45.70 ± 11.95	644.11 ± 113.53	25.47 ± 10.31	108.06 ± 11.73	14.26 ± 1.68	70.03 ± 19.26	27.16 ± 13.35
JA	5.82 ± 2.49	45.46 ± 2.01	9.37 ± 4.78	44.52 ± 4.35	8.85 ± 2.20	50.06 ± 2.41	5.67 ± 1.58	17.37 ± 3.22	46.71 ± 9.33	22.07 ± 0.19
JA-Ile	1.24 ± 0.38	22.47 ± 1.42	1.03 ± 0.51	18.50 ± 1.54	0.79 ± 0.18	21.34 ± 1.61	0.40 ± 0.10	4.26 ± 1.00	11.32 ± 1.36	9.05 ± 0.15
ABA	20.65 ± 1.11	402.43 ± 11.09	10.05 ± 0.66	833.50 ± 30.88	9.51 ± 2.54	813.65 ± 19.38	11.93 ± 1.18	1240.54 ± 156.20	13.89 ± 1.47	947.84 ± 47.04
SA	231.55 ± 22.51	136.50 ± 26.54	256.71 ± 36.30	141.73 ± 34.72	205.85 ± 28.62	154.37 ± 24.52	126.66 ± 6.60	144.64 ± 17.39	140.49 ± 19.39	242.69 ± 70.08
SAGE	4080.07 ± 342.73	3307.61 ± 188.64	2584.74 ± 386.73	3484.22 ± 383.97	4803.43 ± 1023.73	7425.88 ± 753.24	970.90 ± 157.53	3862.16 ± 83.70	2364.91 ± 313.83	5750.87 ± 302.27

Table S2. Hormone content in barley leaves. Hormone content before and after 7 d of drought treatment in leaves of KD Pap1 (1130 and 1175 lines), KDPap19 (1770 and 1776 lines), and control (WT) plants. Data, referred as ng g⁻¹ dry weight are means ± standard error of three independent biological replicates.